CONTRIBUTIONS TO EMBRYOLOGY

VOLUME IV, NOS. 10, 11, 12, 13

PUBLISHED BY THE CARNEGIE INSTITUTION OF WASHINGTON
WASHINGTON, 1916

CARNEGIE INSTITUTION OF WASHINGTON
PUBLICATION NO. 224

PRESS OF GIBSON BROTHERS
WASHINGTON

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THE HUMAN MAGMA RÉTICULÉ IN NORMAL AND IN PATHOLOGICAL DEVELOPMENT.

By Franklin P. Mall.

With three plates.

CONTENTS.

THE HUMAN MAGMA RÉTICULÉ IN NORMAL AND IN PATHOLOGICAL DEVELOPMENT.

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INTRODUCTION.

Students of embryology are familiar with the jelly-like substance found in the human exocœlom, which varies much in appearance in different specimens. Sometimes this substance is gelatinous, with delicate fibers; at other times it is mixed with granules; and, in extreme cases, it forms quite a solid body. I think it was Giacomini who pointed out definitely that the morphological appearance of the magma determines, with considerable certainty, whether or not the contained embryo is normal or pathological. We are indebted to him for about a dozen papers on pathological embryology, a summary of which he published in Merkel-Bonnet's *Ergebnisse*. In this summary the following statement is made:

"In the early stages of development we can determine by the extent of the exocœlom and its contained magma whether or not the embryo under consideration is normal. A large cœlom, containing a rich magma, with its meshes sufficiently filled with a flaky precipitate to mask the embryo, is a certain sign of pathological development."

It is well known that the magma is least conspicuous in fresh specimens and becomes more pronounced after being hardened in alcohol or other preservative fluids. In recent years it is found that magma shows to the greatest advantage in specimens hardened in formalin; the fibrils are somewhat tougher, but the magma has usually the same appearance as in the fresh state. However, the experience of embryologists has been that the magma is more pronounced in pathological specimens, and for this reason it has been suspected that it does not exist in normal development. In fact, the illustration of the magma given by Velpeau in his monograph—in which he first uses the term "magma réticulé"—is undoubtedly of a pathological specimen. A glance at the other plate which accompanies this handsome monograph shows clearly that most of the specimens he describes are decidedly pathological. During the 80 years which have elapsed since his time, embryologists, through comparative study, have been able to separate normal from pathological embryos with considerable precision; and in the abortion material, as collected in various laboratories, far over one-half of the specimens of the first 2 months of pregnancy are pathological, and in them we usually find a highly differentiated magma. However, if normal specimens are studied with care, we find that they, too, contain some magma; therefore, magma must be viewed as a normal constituent of the human ovum.

It has been shown by Keibel that there is marked magma within the exocœlom of monkey embryos. In specimens containing embryos 1.3 mm. and 5 mm. in length, he describes it as a flaky, reticular mass outside the amnion, and speaks of it

as coagulum composed of reticular magma which had to be removed before the embryo could be seen. Undoubtedly he was dealing with normal specimens, thus showing quite conclusively that a delicate magma must be viewed as a normal constituent of the exocœlom. According to Keibel's figure 7, the magma appears to be denser in monkeys than is usually the case in normal human specimens. However, a very dense magma in human specimens invariably indicates, as was first demonstrated by Giacomini, that the ovum is pathological.

The best account of magma réticulé is given by Retzius, who brought the subject up to 1890, and left it with the conclusion that magma réticulé is a normal constituent of the human ovum. The statements of the earlier embryologists, from the time of Haller, are mainly of historical interest; but these investigators were at times inclined to view magma as a "middle embryonic layer" of the ovum, and, again, they believed it to represent the allantois of lower animals. Retzius reinvestigated the subject, taking into consideration normal as well as pathological embryos, and his conclusion is that magma is present in both kinds. His own words are as follows:

"Bei der Oeffnung des Chorionsackes der Eier des ersten und zweiten Monats sah ich, wie in der Einleitung erwähnt wurde, und dies oft, am besten nach kurzer Erhärtung in Ueberosmiumsäure (von $\frac{1}{4}$ Proc.) oder in Müllerscher Lösung (gewöhnlich nach doppelter Verdünnung), in dem schleimigen Inhalt, welcher zwischen dem Chorion und dem Amnion, also im subchorionischen Raume vorhanden war, dünnere oder dickere Fäden und Stränge, die mehr oder weniger dicht von der äusseren Fläche des Amnion zur inneren Fläche des Chorion hinüberliefen, um sich dort mit ihren Enden an den beiden Häuten zu befestigen, indem sie sich oft an ihnen verbreiterten und in ihre bekleidende Schicht übergingen. Diese Fäden und Stränge, welche im frischen Präparate kaum sichtbar waren, traten nach der Behandlung mit den erwähnten Flüssigkeiten deutlich hervor. In der Fig. 15 der Taf. XVIII habe ich ein solches Ei abgebildet. Das stark zottige Chorion (*ch*) ist geöffnet, und man sieht im subchorionischen Raume den Amnionsack (*a*) an weisslichen Strängen (*m*) aufgehangt liegen.

"Die Stränge, welche im unerhärteten Zustande eine schleimigfaserige Consistenz haben, sich aber ohne Schwierigkeit Stückweise ausschneiden lassen und dann in Mikroskope eine deutlich faserige Structur darbieten, zeigen nach der Erhärtung einen ausgeprägt fibrillären Habitus. In einer homogen, structurlosen Grundsubstanz treten Züge echter bindegewebiger Fibrillen hervor, welche oft eine Hauptrichtung einschlagen, also ziemlich parallel verlaufen. Jedoch kommen auch viele sich kreuzende Fasern vor. Hier und da bemerkt man dickere Bündel verschiedenen Calibers welche aus dicht gedrangten Fibrillen bestehen. Es sind also fibrillär-bindegewebige Balken, welche durch eine homogene, zahlreiche einzelne Fibrillen enthaltende Intercellularsubstanz ziehen. Zwischen den Balken und Fibrillenzügen sieht man recht zahlreiche Zellen, welche theils und am meisten rundlich oder oval, theils auch spindelförmig sind und in ihrem oft reichlichten Protoplasma grössere glanzende Korner enthalten. Diese Zellen liegen in der Grundsubstanz ohne besondere anordnung zerstreut, bilden also keine Scheiden o. d. um die Fibrillenbündel.

"Man hat es hier offenbar mit einem *unreifen Bindegewebe* zu thun, einem *embryonalen mucösen Bindegewebe, welches indessen in der Entwicklung zum fibrillaren Bindegewebe schon weit vorgeschritten ist.*"

THE MAGMA IN NORMAL DEVELOPMENT.

We have now in the literature a detailed description of a number of young human ova, and, according to their clinical histories, some of them, at least, must be normal. The classic specimen is that described by Peters, which came from a woman who had committed suicide. The specimen was hardened *in situ* in an approved manner, and was worked up and described under the best possible conditions. In it the cœlom is filled with a gelatinous substance, through which radiate

delicate bands of fibrils, among which appear scattered nuclei. Near the embryo there is a small space, the interpretation of which was very difficult at the time the specimen was described.

Since Peters studied this specimen, the sections have been carefully reworked and discussed in a critical way by Grosser, who gives a new interpretation in two figures and states that the cavity of the ovum contains reticular magma which is partly made up of heavier strands of tissue accompanied by nuclei. In the neighborhood of the embryo there are two large spaces, lined with cells, which appear to be the primitive body-cavities. In his work on the comparative development of the embryonic membranes, Grosser describes this space in great detail and also gives us two new illustrations of the embryo in his plates 3 and 4. According to this authority these two body-cavities communicate by means of a slit-like canal just behind the umbilical vesicle (Grosser's figure 31, plate 4). This interpretation of the Peters specimen shows that the cavity of the ovum is first filled with a free mass of reticular magma, after which the cœlom begins to form near the body of the embryo. As this cavity expands subsequently, it probably first destroys the more delicate strands of magma, leaving the heavier ones; thus in a short time the cavity of the ovum is lined by the endothelium of the cœlom, which also must cover the stronger bands of magma radiating as trabeculæ throughout this cavity (Grosser, pp. 78 and 79).

Keibel explains the formation of the human cœlom as follows:

"It is, however, not quite clear how the cavity traversed by scattered strands of mesoblast and lying between the yolk-sac and the chorion in the Peters ovum is to be interpreted. It may be supposed to represent the extraembryonic cœlom; but it may also be imagined that it has arisen from an extensive loosening up of the tissue, and not by a splitting of the mesoderm, and that the triangular space between the caudal extremity of the embryo, which is lined with flat cells having an epithelial arrangement, is the first primordium of the cœlom."

A condition similar to that found in the Peters specimen has been observed by Lewis in the Herzog specimen, which is of about the same stage of development. Lewis says (see his paper, p. 300) that there are occasional clefts in the mesoderm of the chorion of the Herzog embryo, but that they are of doubtful significance. His reconstruction shows a strand of mesoderm, more pronounced than in the Peters ovum, extending from the yolk-sac to the chorion and circumscribing a space on the ventral side of the embryo.

Eternod has written several papers in which he describes the formation of the exocœlom and the fate of the magma réticulé. He says that it first fills the entire space between the primordium of the embryo and the chorionic wall. Later, larger spaces appear within the substance of the magma, leaving denser strands of magma fibrils to support the embryo within the gradually expanding chorion. In general this coincides with the opinions just cited.

The relation of the exocœlom to the magma is strikingly shown by Waterston in a section of a small embryo *in situ*. The space between the embryo and the chorion is filled with a dense mass of fibrils, into which the exocœlom is burrowing. Waterston's figure 1 shows the relation of this cavity to the magma, and only near the embryo is the exocœlom lined with a layer of cells. When this figure is compared

with Grosser's figure of the Peters ovum, it becomes clear that the two spaces in the latter are in reality the beginning of the exocœlom.

The studies referred to above indicate that the space near the embryo is the primitive exocœlom and that the remainder of the so-called cavity of the chorion is simply the young normal ovum filled with delicate fibrils which communicate freely with the fibrils of the chorionic membrane. We have in our collection a young normal specimen, No. 763, containing an embryo anlage 0.2 mm. in length, which in general confirms the observations in the Peters ovum. A list of the normal specimens in our collection discussed in this paper is given in table 1.

TABLE 1.—*List of normal embryos.*

Cat. No.	Length of embryo	Dimensions of chorion.	Menstrual age in days.	Condition of magma.	Cat. No	Length of embryo.	Dimensions of chorion	Menstrual age in days.	Condition of magma.
	mm.	*mm.*				*mm.*	*mm.*		
763	0 2	4× 2 2	60	Some reticular.	588	1	19×15× 8	49	Strands of magma.
391	2	16×14×12	14 (?)	Do.	136	4	14×11× 6	56	Reticular excessive.
779	2 75	16×14×12	40	None.	836	4	22×18×11	36 (?)	Delicate reticular.
164	3 5	17×17×10	.	Few strands	148	4 3	17×14×10	31	Small amount of magma around cord.
463	3 9	17×12× 7	48	Much reticular.					
486	4	22×22×22	41	Do.	576	17	30×30×25	...	Small amount of magma.
470	4	20×13	34	Very few fibrils.					

Specimen No. 763 was removed from a woman who was the mother of 6 children, the oldest being 10 years old. She had had one miscarriage. During the year before the operation she suffered much from headache and backache, but otherwise her health appeared to be normal. When she was admitted to the hospital she complained of abdominal enlargement and there was some urinary disturbance. At the operation for rupture of the perineum the uterus was scraped out; subsequently the ovum was found in one of these scrapings. The fragments both of the mucous membrane and ovum appear to be normal.

Unfortunately we have only a few of the sections of this valuable specimen, but these show that we are undoubtedly dealing with a normal ovum of the same stage of development as that described by Peters. The chorionic cavity is partly filled with mother's blood, but there are some strands of reticular magma, with nuclei and protoplasm radiating through the blood. The specimen has been stained in hematoxylin and eosin, which is not especially favorable for defining magma fibrils.

The specimen described by Herzog is also undoubtedly normal, as it was obtained from a woman who was killed by a stab-wound through the heart. The large colored plate published by Herzog shows the specimen to be quite identical with that of Peters. It shows free cells in the cœlom, which contains no other foreign substance, but a photograph (figure 24, published by Herzog) shows that the cœlom is filled by a very pronounced substance, reminding one very much of reticular magma. The same is true of a specimen recently described by Johnstone. A colored photograph which he published shows quite distinctly a pronounced magma throughout the cœlom. (See, for instance, his figure 3.) This establishes definitely the presence of reticular magma in ova the size of the specimen of Peters. We have, however, the valuable specimen of Bryce and Teacher, which also shows the condi-

tion of the magma in an earlier stage. In this specimen the chorionic cavity is filled with a dense mass of fibrils, throughout which are scattered numerous nuclei, as shown in their plates 3 and 4. The specimen was not perfectly hardened and there is a small cleft between the chorionic wall and the mass of magma. As yet there is no exocœlom, showing that it is younger than the Peters specimen.

More advanced stages of the condition of the magma are represented in the specimens described by Jung and by Strahl and Beneke. In the Jung specimen the cavity of the ovum is filled with a very pronounced magma, running together in stronger bands, as in our own normal specimen, No. 836, to be described later. The larger cavity Jung marks "exocœlom," but it is not clear that this is lined with endothelium. From his large illustration one gains the impression that the specimen is somewhat pathological, for it is of the same type as numerous specimens in our collection with embryos that are usually found to be pathological. Taking the illustrations given in Jung's plates 1 and 2, the specimen again appears to be pathological, and I should be inclined to pronounce it such did not his plate 6, figure 17, show this same section on an enlarged scale, which gives a very sharp outline of different embryo structures and scattered through them are numerous cells undergoing division. It would be impossible, with our present knowledge, to accept such sections as coming from a pathological embryo. The specimen described by Strahl and Beneke is of about the same stage as the Jung specimen, although the magma does not seem to be so well pronounced. It is unequal in mass and has scattered through it delicate strands, as shown in their figure 63. In fact, the above-described specimen underlies also the diagram on the form of the cœlom given by Strahl and Beneke on page 18 of their monograph.

Magma of uniform consistency, as seen in the Bryce and Teacher specimen, soon arranges itself in bands, which gradually become more and more pronounced in older specimens. Between these bands are spaces filled with fluid, and those spaces near the embryo become lined with endothelium to form the exocœlom. There are other spaces between the exocœlom and the chorionic wall. The sharper bands of magma fibrils—well shown in our embryo No. 836 (plate 1, figs. 3 and 4)—apparently support the embryo and the wall of the exocœlom within the chorion.

We have in our collection an excellent embryo, No. 391, which is a little larger than that described by Strahl and Beneke. This specimen came to us in formalin and was opened with great care. It was found that the embryo and appendages were suspended by means of numerous delicate fibrils which radiated from them to the chorionic wall. As the sections were stained with cochineal, the fibrils do not show in them, so that this description is based entirely upon the apearance of the uncut specimen. In general the specimen appears to be normal.

Our specimen No. 779, somewhat older than the one just mentioned, apparently contains no magma. It also was hardened in formalin. The ovum is entirely covered with villi, which branch twice, are of uniform size, and appear to be normal. In the main chorionic wall there is a pronounced fold. The specimen was bent along the line of the fold, but the chorion was gradually dissected away with the aid of direct sunlight. The chorion is entirely lined by a smooth membrane, and contains a cavity which is filled with a clear fluid and which apparently contains

no magma. Within there is a clear, worm-like body, which is bent upon itself, with another body arising from the middle of the bend. Apparently this is a flexed embryo with the umbilical vesicle attached to it. The body is of uniform diameter, measuring less than a millimeter. We are probably dealing here with a normal embryo. In opening this specimen great care was taken not to touch the embryo, so as to avoid injuring it. The embryo was taken out and cut into serial sections. It contains 14 somites and is without limb-buds. The sections give the impression that the embryo is pathological. There are no data in the history of the case which bear upon this point; therefore, for the present we may view it as a normal specimen without magma—or, if the embryo is taken into consideration, as a pathological specimen with dissolution of the magma. Usually in pathological specimens the magma is greatly increased in quantity.

No. 164 is a somewhat older specimen. It came to us from an autopsy, with the entire uterus, and the sections of it indicate that the embryo is undoubtedly normal. The only record of the magma which we now have is given by several photographs which were taken at the time we received the specimen. These show a few strands of reticular magma, without any granular magma, radiating from the embryo. The photographs were taken while the specimen was in formalin.

The next specimen, No. 463, is somewhat more advanced in development and contains a flexed embryo, 3.9 mm. in length. The ovum is covered completely on one side, and partly on the other, with villi 1.75 to 2.75 mm. long. On the partly covered side the villi leave relatively bare one area, centrally situated, measuring 8 by 4.5 mm. Over it the villi occur only here and there, about 2 mm. apart, and are branched and apparently normal. On opening the ovum the reticular magma is found to fill the exocœlom. By carefully exploring with fine tweezers, an apparently normal embryo is seen with a yolk-sac measuring 3.5 by 4 mm. The embryo has anterior limb-buds and at least three gill-slits which are visible externally. No note was taken at the time regarding the condition of the magma, but sections of the entire chorion show that there is a very decided reticular magma between the embryo and the chorionic wall. There is no granular magma. The magma is composed mostly of fibrils, of much the same appearance as those of mesenchyme. Between the network of magma fibrils are denser strands accompanied by cells. In the fresh state undoubtedly the denser strands would appear as fibrils, while the rest would be transparent and jelly-like. The specimen came from a woman who was perfectly healthy and had given birth to 2 children during the last 4 years. This was her first miscarriage, and there was no indication of uterine disease.

Specimen No. 486, of the same degree of development as the one described above, is in a perfect state of preservation, but there is no history which would indicate whether or not the specimen is normal. However, the chorion is covered with villi about 3 mm. long, with a bare spot on one side about 4 mm. in diameter. The sections of the embryo do not show any attached fibrils of magma, but the chorionic wall, after hardening in alcohol, shows a decided layer of magma attached to it.

No. 470 is an interesting specimen, as it was found floating in a mass of blood-clots, which were sent to the laboratory in formalin. The ovum is covered with

normal villi and contains a well-formed embryo within the amnion. It is apparently normal in every respect. No magma could be seen at the time, but drawings of the embryo subsequently made show delicate strands of fibrils forming a fuzzy layer around the umbilical cord and extending over the umbilical vesicle; undoubtedly these are magma fibrils. This seems to be the normal condition for this stage and is verified in specimen No. 836, to be described later. Sections through the mass and the chorion, stained with carmine, show the magma as a granular mass; only at points is there any indication of fibrils. However, this mass resolves itself into the most definite fibrils when colored with Van Gieson stain, in Mallory's stain, in hematoxylin, aurantia and orange G., or in iron hematoxylin. With Van Gieson stain the fibrils take on fuchsin color about as intensely as do the fibrils of the chorionic wall, with which they are continuous. The contrast obtained with Mallory's stain is quite marked, as the endoplasm of the mesenchyme of the chorionic wall stains slightly blue, while the exoplasm and the fibrils of the magma réticulé remain unstained. This difference is not shown in sections stained in iron hematoxylin, as all fibrils are colored intensely black. However, it does not come out with the Oppels-Biondi method or with hematoxylin and eosin or aurantia. As the fibrils of the magma are continuous with those of the exoplasm of the chorionic wall, which do not stain in Mallory's connective-tissue mixture, they can not be considered as white fibers, and from their failure to stain in Weigert's elastic-tissue mixture they are not elastic. As will be shown subsequently, they give the reactions of embryonic connective-tissue syncytium; and this is Retzius's opinion regarding their character. In specimen No. 486 the fibrils of the magma are not accompanied by any nuclei; so they must be viewed as belonging to the cells of the chorionic wall, from which they extend to bind the chorion with the primordium of the embryo.

Specimen No. 588 came from a woman who had 2 children living, aged 14 and 20 years respectively. Since the last birth she had aborted 11 times, and in the opinion of her physician all the abortions were due to mechanical means. This indicates that the specimen is normal. A figure of this embryo with strands of magma radiating from the umbilical cord and vesicle is shown in plate 3, figure 2.

Specimen No. 136 is of about the same stage of development as No. 588, although the chorion is covered with poorly defined villi. For an embryo of this stage it is unusually small, and I have therefore listed it with the pathological specimens in my paper on monsters. A photograph of the ovum after it had been cut open shows that the chorion is completely filled with reticular magma, so that the embryo is practically obscured. A block of the whole ovum encircling the embryo was cut in serial sections. These show that there are strands of tissue accompanied by cells which form partitions in the exocœlom. The quantity of the magma appears to be somewhat excessive for a normal ovum of this stage of development.

No. 836, a perfect specimen containing an embryo 4 mm. in length, settles definitely the condition of the magma at this stage of development (plate 1, figures 3 and 4). In this ovum the exocœlom, measuring 9 by 4 mm., contains a delicate spiderweb-like reticular magma, several of the strands being considerably larger than the others. Most of this magma occurs between the yolk-sac and the amnion

and the adjacent chorionic wall where the fibrils are unusually abundant. This specimen was obtained from a hysterectomy upon a woman, 25 years old, for a fibrous tumor of the uterus. She had been married 4 years, this being her first pregnancy. There were no special symptoms bearing upon the case, excepting the discomfort which accompanied the tumor of the uterus. Her last menstrual period had been delayed, and as it had been more profuse than usual she believed that she had had a miscarriage; otherwise, everything appeared normal. This was confirmed by a careful examination of the specimen, which showed it to be normal in every respect. The uterus was opened by the surgeon at the time of the operation, but fortunately the site of the ovum was not injured. The specimen was sent to the laboratory immediately, where it was fixed by Dr. Evans, who made the following record:

"The specimen consists of a myomatous uterus which has been opened (apparently in a midline anterior incision) so as to disclose an abundant deciduous endometrium thrown into large folds. At the upper posterior surface of the uterus an oval mass, about 25 by 20 by 20 mm., projects. It is a sac and is covered with a rather smooth membrane (decidua reflexa), beneath which tortuous vessels are apparent. On one side the sac (the implanted chorion) is adherent to the uterine mucosa (decidua vera). With a sharp scalpel the entire mass was dissected away from the uterus and brought under a binocular microscope in warm salt solution. The middle portion of the free surface was opened carefully, beautiful villi being found, and then the delicate wall of the chorion was divided. Within, a transparent young embryo and its umbilical vesicle were seen, the embryo appearing to be about 5 mm. in length. Through this opening in the chorion, warm (40° C.) saturated aqueous solution of $HgCl_2$, containing 5 per cent glacial acetic acid, was gently introduced and the entire mass placed in 500 c.c. of this fixation fluid. The main body of the uterus was dissected from the myomatous nodule and fixed in 10 per cent formalin, the site of the implanted ovum being marked by a short wooden rod."

The fixed and hardened specimen had undergone a readily appreciable shrinkage from the condition seen in warm salt solution. All of the tissues were beautifully preserved. The implanted ovum, covered with the decidua capsularis, measures approximately 22 by 18 by 11 mm. The adjacent decidua parietalis is thrown into large folds, which are themselves marked by numerous tiny elongated crack-like depressions, as well as by more circular pit-like apertures. The relatively smooth but irregular surface of the decidua capsularis is marked here and there by very conspicuous, small, oval pits, which may attain 0.5 mm. in diameter. The four flaps of this coat at its highest point, where it was opened directly over the middle of the ovum, are rather smooth on their inner surface and stand apart from the subjacent chorionic villi (intervillous space) to which they were originally adherent. The villi are about 2.5 mm. in length and possess one or two large branches and many "stub-like" tiny bulbous ones on the main stem. The villi are uniformly distributed in the small area exposed. With a slender scalpel the ovum was carefully divided under the dissecting microscope, the embryo and yolk-sac being visible. The yolk-sac appears to be almost 2 cm. in diameter and the embryo is surrounded by its amnion, its head (visible from above) being about 3 cm. in length and showing the fourth ventricle covered by a transparent ependyma. Two gill-arches are visible. The yolk-sac surface presents an exquisite picture of irregular, clear vascular channels and a uniform pattern of small, opaque, white blood-islands. The preservation seems perfect.

After the embryo had been carefully removed, the ovum was cut into blocks which included its implantation. A block 1 mm. thick, which included the largest circumference of the embryo, was embedded in celloidin, the sections being stained in various ways. A photograph of this block is represented in plate 1, figure 4, which shows strikingly the extent of the magma. Sections which have been stained in hematoxylin and aurantia show the magma much as it appears in the other embryos that have just been considered. There is a denser magma just under the chorionic wall, and heavy strands radiate in every direction, with a fine network resembling spider-web, among the main strands. A number of loose nuclei accompany these strands, but they do not have the appearance of the nuclei of the main wall of the chorion. They are mostly round and are of unequal thickness, simulating very much the blood-cells. Occasionally there is a large nucleus. Sections which have been treated by the Weigert fibrin method do not show these fibrils. This confirms a previous experience which I have published elsewhere in my paper on monsters, namely, that magma fibrils do not give the reaction of fibrin, nor do these fibrils stain well in Van Gieson's mixture; however, they take on color similar to the mesenchyme of the chorion.

At points it appears as though these fibrils arise directly from the chorionic wall. They stain intensely blue by the Mallory method, and in sections treated in this way the nuclei of the mesenchyme of the villi look much like the accompanying nuclei of the magma fibrils. On one side of the ovum a denser mass of the magma is directly continuous with the mesenchyme of the chorionic wall. However, just in this region the magma contains no nuclei. It, therefore, appears that the magma fibrils must be associated, at least partly, with the nuclei of the chorionic wall. Exceedingly good histological pictures were obtained from sections stained by Heidenhain's method, which show all the transition stages between magma containing no nuclei and magma very rich in nuclei. It would seem that there is quite a free wandering of the nuclei along the magma fibrils, and whenever they come in contact with the chorionic wall the fibrils enter it, showing direct continuity. The most instructive specimens are obtained by the Weigert elastic-tissue stain, which gives a slight blue-black tinge to the mesenchyme fibrils of the chorionic wall, as well as to those of the centers of some of the villi. The magma itself takes on a very light stain, but where it is in contact with the chorionic wall it grades over into its blue network. It appears, then, that the centers of the villi, which represent their older portion, stain somewhat with elastic-tissue stain; and, if we view the chorionic wall as the more differentiated portion of the chorion, we must conclude that the older mesenchyme fibrils behave more like elastic-tissue fibrils than do the younger. At any rate, the magma fibrils do not take on elastic-tissue stain.

From all that has been said it is clear that the mesenchyme of the chorionic wall and the magma fibrils are continuous and, as I have pointed out elsewhere, they together form a common syncytium. I have already demonstrated that very young connective tissue arises directly from the mesenchyme, the earlier stages of which I have designated as the connective-tissue syncytium. Towards digestive reagents the connective-tissue syncytium gives somewhat the reaction of yellow elastic tissue, just as do the mesenchyme and the magma of No. 836 when treated with Weigert's

elastic-tissue stain. I have also shown that the younger the connective-tissue syncytium is, the more difficult it is to digest it in pepsin. Frozen sections shrink but little when treated with acetic acid, while white fibers become transparent. The syncytium itself is somewhat elastic, as shown by pressure upon the cover-glass over a frozen section. If treated for 24 hours with pepsin, the fibrils disintegrate. They are therefore much more resistant to the action of pepsin than are white fibrils.

The action of pancreatin is, in a measure, the opposite of that of pepsin. When the main mass of syncytium is formed by exoplasm, it digests readily in pancreatin. The more the syncytium is developed, the more resistant it is towards pancreatin. Very young syncytium fibrils, therefore, react towards pancreatin and pepsin much like elastic fibers and this is confirmed in a measure, by tinctorial methods, when applied to sections of the chorion and magma, in specimen No. 836.

I have discussed the denser strands of tissue within the main mass of the magma. In the fresh state it appears that these are distinct fibrils, as shown in plate 3, figure 2. They are, also, observed in plate 1, figure 3. It is not quite so clear that there are fibrils in the magma as shown on plate 1, figure 4. In fact, it appears as though we have compartments separated by membranes, and that at the junction of several of these membranes the fibrils become denser, and therefore often appear as distinct fibers. It would be more appropriate than to state that the exocœlum is broken up into compartments the walls of which are composed of membranes, and that where several of the membranes come together the increased amount of tissue gives the point of juncture the appearance of fibers to the naked eye and under the enlarging lens.

I have taken great pains to follow the cells which mark the stronger bands of magma, and it is difficult to arrive at any conclusion, for, in a measure, they seem to be related to the endothelial lining of the exocœlom. In the Peters ovum the spaces near the embryo are lined by a distinct layer of cells, but otherwise there is no indication of endothelial lining in any other portion of the chorionic cavity, nor is there any indication of such a lining in the figures given by Herzog, Johnstone, Jung, or Strahl and Benecke. It would seem that what corresponds to the exocœlom of the chorion in the later stages is represented by a diffuse mass in the specimen of Bryce and Teacher where the nuclei are scattered through it. The mode of the destruction of the mesenchyme is well indicated in figures on page 18 of a monograph by Strahl and Benecke. These irregular cells are first of all attached to the heavier strands of magma, and they must, therefore, correspond to the endothelial lining of the exocœlom. For the present, however, it appears as if the exocœlom of the human chorion is lined only in part by a layer of endothelium; these cells also accompany the magma fibers and line the inner side of the chorion near the embryo.

As the amnion expands, it naturally pushes these strands of magma up against the chorion, and in a short time we can recognize only a few fibrils in the exocœlom which encircle the umbilical cord. These are well seen in specimen No. 148, and their remnants are shown in No. 576, of which I give an illustration on plate 2, figure 2. No. 148 is undoubtedly normal, for it was obtained by mechanical means, and No. 576 is also a normal specimen obtained from a tubal pregnancy.

The conclusion regarding the condition of the magma of normal development is that the cavity of the ovum is filled with delicate fibrils which are interpersed with numerous nuclei and which form one continuous network, extending from the embryo to the chorionic wall, and blending with its connective-tissue network. It forms one continuous syncytium, and as the ovum grows the magma réticulé differentiates somewhat. Stronger bands of membranes soon form, breaking the cavity of the chorion into compartments. This process continues until the amnion begins to expand, and then these fibrils are pushed up against the chorionic wall. The exocœlom begins as two larger spaces near the embryo, and in this portion of the ovum its cavity is lined with a layer of endothelium. It is quite certain that this sharply defined cavity does not extend to include the whole cavity of the ovum, but the cells lining it arise in common with those which accompany the magma fibrils. The exact extent and the fate of the two small spaces near the embryo in the Peters specimen is still undetermined, but Waterston's specimen indicates that they do not extend to fill the entire chorionic cavity. The examination of numerous specimens, however, indicates very definitely that the exocœlom of the ovum at 2 months does not contain a complete endothelial lining.

THE MAGMA IN PATHOLOGICAL OVA.

Since the publications by Giacomini it has become well known that an increased quantity of magma within the cœlom indicates with certainty that the embryo is pathological. When the magma is pictured or described, it is quite easy to determine whether or not the embryos and ova published in the literature are normal or pathological. This is demonstrated in the plates accompanying Velpeau's work. His was able to separate most of the normal from the pathological embryos, but he relied mainly upon the external form of the specimens, which he compared with other mammalian embryos. Unless an embryo appeared much like those of other mammals and was not transparent and sharply defined, he decided that it was not normal but pathological. The work of Hochstetter, who limited his study to embryos obtained through hysterectomy, has been used to advantage by Keibel and Else in the preparation of their Normentafel, so that now we have adequate tables and plates which enable us to recognize with considerable certainty whether or not an embryo is normal, without paying much attention to the magma or the chorion. However, embryologists are well aware that they can predict whether a specimen is normal or pathological by the quantity of the magma which masks the embryo when the ovum is opened.

By the contents of the exocœlom it is quite easy to classify pathological ova into three chief groups. In the first group, which includes most pathological specimens, the magma is changed into an organized mass of reticular fibrils, intermingled more or less with granular substance.

To the second group belong specimens in which the exocœlom is large and contains only a fluid mass—that is, a liquid substance which does not coagulate in either formalin or alcohol. I have pictured a number of specimens of this sort in my paper on monsters. Specimen No. 512, of which I give an illustration on plate 2, figure 1, belongs to this group. The embryo is atrophic, and it is questionable

whether or not it is encircled by the amnion. In these specimens the cœlom is usually enlarged and sometimes it is greatly distended. Often there is a small granular precipitate in older specimens, but this is not of sufficient quantity or density to form a continuous mass. The histories of these specimens show that they are considerably older than their sizes indicate, and I am inclined to view them as having once had a dense mass of magma within the cœlom, which subsequently underwent dissolution, leaving a more or less flaky deposit that finally disappeared altogether.

In the third group, the cœlom is greatly distended, the amnion is usually absent, and the ovum is filled with a gelatinous substance. This is well illustrated by specimen No. 604, plate 1, figures 1 and 2.

TABLE 2.—*List of specimens containing pathological magma.*

Cat. No.	Length of embryo.	Dimensions of chorion.	Menstrual age.	Contents of ovum.	Cat. No.	Length of embryo.	Dimensions of chorion.	Menstrual age.	Contents of ovum.
	mm.	*mm.*	*days.*			*mm.*	*mm.*	*days.*	
278	.	. . .	.		512	10	30×27×18	. . .	No magma.
660	.	40×35×30	67		636	10	28×28×22	56	
813	.	80×50×25	200	Hyaline magma.	605	[10 5]	45×10×25	. . .	
78	1	36×35×13	87	Fluid.	101	12	35×35×15	35	Granular-hyaline; also no magma.
531	1 5	19×19×19	15	Flaky.	. . .	. . .	. . .	. . .	
250	2	10×9×8	.		211	12	. . .	. . .	
12	2 1	18×18×8	11		1117	14	. . .	.	Hyaline magma.
318	2 5	20×18×11	42		270	14	40×30×20	.	Granular; hyaline also.
543	3	70×30×25	77		. . .	. . .	. . .	. . .	
651*a*	3	35×30×30	.	Granular.	991	17	. . .	73	
244	1	25×15×15	.	Fluid.	604	17	70×50×50	. . .	Hyaline magma.
402	1 5	10×25×20	42		94	20	.	14	
122	5	20×16×16	65	Do.	1189	22	50×50×50	91	Granular; hyaline.
533	5	35×30×30	56		584*a*	25	50×42×40	. . .	
545	5	12×9×9	53		79	33	50×50×50	.	
21	5 5	12×9×5	. . .		230	57	75×60×50	.	Do.
560	7	. . .	49		261	90	120×70×70	. . .	Do.
135	9	105×65×65	.	Hyaline magma.	. . .	. . .	. . .	. . .	

I will now review several specimens illustrating these three varieties of pathological magma. The specimens considered are arranged in table 2. The list could easily be increased to several hundred, but as the specimens with catalogue numbers less than 403 have been published in detail with illustrations in my monograph on monsters, I will allude only to some of them. The pathological specimens with numbers over 402 are being prepared for publication, so that a few selected specimens with higher numbers are illustrated. Pathological specimens from tubal pregnancy with numbers up to 1,000 will be found described in detail in my monograph on tubal pregnancy.

The first specimen which I shall consider (No. 278) consists of an entire ovum, measuring 6 by 4 mm. It was sent me by Dr. Stanton, of Albany, New York. The specimen might be viewed as normal, but it contains no embryo, and as it was obtained from a diseased uterus, it is probably pathological, the magma having undergone minor changes.

This ovum was found accidentally in curettings from a woman supposed to have chronic endometritis following pregnancy. There is nothing in the history from which the age of the specimen could be estimated. Part of the specimen had been

cut into sections before it was received at the laboratory, with the statement that no embryo had been found, it having fallen out. I found that the half sent contained a cœlom, 3 by 2.5 mm., filled with magma, in which there was a cavity about 1.5 by 1 mm. Sections showed that the cavity was natural and not sharply defined, with nothing to indicate that it had contained an embryo. On the contrary, it was found that the magma réticulé was composed of a loose network of mesoderm cells, which bound one side of the chorion with the other. These cells are directly continuous with those of the mesoderm and resemble them in every particular. At one point there is a small group of epithelial cells, which may represent what was originally the embryo. Otherwise, the chorion and its villi are normal in appearance, being encapsulated in decidua which has in it some uterine glands. All in all, this specimen reminds one very much of the Peters ovum. There are some leucocytes in the decidua, but no accumulation of them indicating inflammation of the uterus. Several figures, illustrating this specimen, may be found in my monograph on monsters.

Specimen No. 531 is in many respects similar to the one just described (No. 278). It came from a woman who had been pregnant 6 times, her periods having been 17 days overdue before this abortion. The ovum is spherical, 19 mm. in diameter, and is covered only by a mass of villi, which appear normal. The cœlom within contains many magma fibrils, the meshes of which are more or less filled with dense granules, as is shown in plate 1, figure 8. Within this mass there is a detached vesicle, 1.5 mm. in diameter, which no doubt represents the umbilical vesicle.

A specimen intermediate between the two just described is No. 250, of which several illustrations are published in my paper on monsters. The specimen came embedded in a mass of decidua and was obtained by scraping the uterus. When opened it was found filled with magma réticulé just beneath the chorion, in which could be seen a small embryo, and farther away towards the center of the cœlom was the umbilical vesicle. The whole ovum was cut into sections. The chorion and the villi are apparently normal in shape and structure, being also rich in blood-vessels, which are filled with embryo blood. The villi are bathed in mother's blood and covered with an active trophoblast. The decidua is somewhat infiltrated with leucocytes, but there are no abscesses. The front end of the amnion is absent, and its free edge and the embryo are embedded in reticular magma, indicating that the amnion was destroyed before the abortion took place. The general shape of the embryo and its degree of development are practically normal. The heart is well formed and, including the blood-vessels, is filled with blood. The alimentary canal, brain, spinal cord, otic and eye vesicles, myotomes, and branchial arches are much like those of embryo No. 12, to be described presently. The septum transversum is well marked and the thyroid gland is just beginning. The tissues of the embryo, however, and the cavity of the front end of the brain are filled with numerous small round cells with fragmented nuclei. All stages of fragmentation are seen, just as may be observed in the leucocytes in small abscesses. Most of the red blood-cells are within the blood-vessels, but those within the tissues appear perfectly normal. On account of the diminished number of mesoderm cells, which, in fact, diminish in proportion as the fragmented cells increase, the con-

clusion must be drawn that the fragmented cells arise from the mesoderm cells. The epidermis covers the whole embryo. The primary change in this specimen is no doubt in the mesoderm, for all the rest of the embryo appears normal. That the equilibrium was overthrown is indicated by the necrotic amnion and the great amount of reticular magma in the exocœlom. What is especially interesting in this specimen is the partial destruction of the amnion, which brings the embryo directly in contact with the pathological magma of the cœlom.

Embryo No. 12, which has been just referred to, may also be discussed in this connection. It was questionable for a long time whether or not the embryo was normal, as the villi and contents of the cœlom and embryo are beautifully preserved and show no pathological change. However, more careful consideration of the specimen shows that there are a few fibrinous masses between the villi, with every indication of uterine inflammation and infection. The extent of the reticular magma is more pronounced than usual, and it was necessary to dissect it away before the embryo could be isolated sufficiently so that it could be well seen. The head is no doubt atrophic, and I am fully convinced that this part of the embryo must have undergone pathological changes a short time before the abortion.

Specimen No. 318 is much like No. 250. The ovum, measuring 20 by 18 by 11 mm., is covered with villi which appear to be perfectly normal. Upon opening, it was found to be filled with stringy magma, on one side of which was embedded an embryo 2.5 mm. in length. The head is sharply outlined, but the embryo seems to continue directly with the umbilical vesicle, leaving an atrophic tail. Sections show that the amnion over the head has dissolved, leaving a picture very much like that shown in No. 250. We have here a small embryo with a very large cœlom, the ovum being moderately filled with reticular magma and a small embryo only partly covered with the amnion. No. 543 is another embryo of the same type. The magma is a little denser than in No. 318. The chorionic villi are developed, but markedly pathological, as the photograph shows. The embryo within is 3 mm. long, lying quite free within the mass of magma. It is covered by a ragged amnion; that is, the amnion is partly destroyed.

An interesting specimen in this connection is No. 402, which is partly described in my paper on monsters, since the issue of which the embryo and chorion have been cut into serial sections. The villi of the ovum are not well developed, and they are distributed irregularly over the surface. The cœlom is filled with reticular magma. The embryo is club-shaped, the head being much too far advanced for the body. The umbilical vesicle is normal in size; the heart is well outlined, and the extremities are just appearing. Sections show that the amnion is greatly distended. Sections of the chorion were stained with cochineal and Van Gieson, and show beautifully the fibrillated structure of the chorionic membrane. These fibers take on red stain, as do those of the reticular magma. The two are continuous, as shown by the illustration on plate 3, figure 3. In fact, this continuity is much more pronounced in pathological than in normal specimens.

Specimen No. 533 (plate 2, figure 3) shows a more advanced stage of an extensive development of reticular magma. The villi of the ovum appear to be normal and the reticular magma is very dense. Between the meshes there are a number of

opaque nodules about 0.5 mm. in diameter. With much difficulty the embryo was teased out, but it was practically impossible to clear it entirely of the magma fibrils. The embryo is long and slender, looking more like that of a dog than a human specimen, the head being unusually small and thin for a human embryo of 0.5 mm. long. The fibers are irregularly stuck together by small granules, and there is a gap in the center which represents the place in which the embryo was located. The illustration shows this condition beautifully. The specimen was sent me by Dr. Fewsmith, of Trenton, New Jersey, who obtained it from a woman whose menstrual period had been a month overdue.

An extremely interesting specimen is No. 545, well illustrated in figure 1, plate 3. The magma is not extensive, but it is pronounced. The embryo is atrophic, and the chorion is only partly covered with villi. The specimen was sent me by Dr. Rand, of New Haven, Connecticut. It was obtained from a woman who is the mother of one healthy child. The last menstrual period began on September 2. Bleeding began on October 22 and ended with the abortion on October 25. The ovum was found embedded in the clots of blood attached to the cervix of the uterus.

An extreme case of degeneration of the magma is shown in No. 660, also well illustrated in figures 4 and 5, plate 3. There is a tendency towards membrane formation, tough strands of fibrils, spaces, and clumps of granules. The chorionic wall is hemorrhagic and degenerated; within there is a collapsed amnion containing a cheesy granular mass.

I shall use two more specimens to illustrate the nature of granular mass in more advanced stages. The first is No. 605 and the second is No. 584*a*. No. 605 is a white transparent specimen, covered with a uniform layer of villi which branch two or three times. The entire specimen measures 45 by 40 by 25 mm.; a small patch of decidua adheres to the outside. The interior is partly filled with coarse strands of reticular magma, having numerous granules attached. On one side of the specimen the umbilical cord is seen, surrounded by a ragged amnion. The tip of the cord has a piece of intestine and stomach hanging from it. The larger masses of tissue which are intermingled with the reticular magma must be the remnants of the embryo, parts of which appear to be normal, and judging from the form and size of the arms and legs the embryo is about 10.5 mm. long. The second specimen is unusually interesting because it contains a normal embryo with hernia of the liver. The exocœlom is unusually large and is filled with a more extensive layer of reticular magma than should be found in an ovum containing a normal embryo of this size.

The remaining three specimens are given because they well illustrate various degrees of reticular magma within the ovum.

No. 560 (plate 1, figure 6) shows very pronounced reticular magma intermingled with much granular. Two stages of somewhat later development are given in Nos. 636 and 991. In the former (plate 1, figure 10) the magma is more pronounced than in normal development, and in the latter (plate 1, figure 7) it is in an extreme amount.

Finally, a unique specimen (No. 1189) throws some light upon the formation of the reticular magma. The ovum came to us within the uterus, having been removed by an operation. At first it seemed to be normal, but on opening it the

embryo was found encircled by a large mass of transparent, tough, stringy reticular magma, which was removed only with great difficulty. It behaved much like the vitreous humor of the eye. On account of its great quantity we at once suspected that the specimen was pathological, and after the embryo was removed it proved to be so. Although quite advanced in development, its head was found to be smaller than normal, the tissues of the face were dissociated, and the borders of the eye were not sharp but ragged. No doubt the specimen had continued to develop normally until shortly before the operation, and the magma increased in quantity and became tough and fibrous. It is an interesting specimen, showing changes in the magma late in development. Sections of the implanted ovum have not yet been made. The specimen is from a negress, 45 years of age, who had had 9 previous pregnancies. Her last menstrual period was 67 days before the operation. Pregnancy was suspected before the removal of the uterus, but a hysterectomy was performed because her periods had become very severe, lasting 8 days and causing faintness and weakness.

The two types of degeneration which the reticular magma undergoes have been considered above. The magma becomes granular and denser as it lessens and becomes liquid. The liquid again either coagulates or remains fluid when the specimen is fixed in formalin. The two fluid types may be related to the destruction of the amnion, but as yet I have been unable to reach a conclusion regarding this point.

The beginning of the formation of granular magma is shown in specimens No. 560 and 991 (plate 1, figures 6 and 7) as well as in Nos. 533 (plate 2, figure 3) and 660 (plate 3, figure 5). It appears to extend into the cavity of the amnion, and often forms great crusts, which surround the embryo, as shown in several specimens pictured in my monograph on monsters (*e. g.*, Nos. 79, 94, 104, 230, and 261). An extreme specimen of granular magma within the exocœlom is shown in specimen No. 651*g* (plate 1, figure 9).

It is extremely difficult to determine with certainty the structure of the granular magma, but in studying pathological ova (especially those obtained from tubal pregnancy) I have frequently observed that there are large masses of granular magma which take on hematoxylin stain. These granules are mixed with a slimy mass which also takes on hematoxylin stain. My attention was called to these granules because they have a characteristic circular stratification and contain within their centers small granules which also stain intensely. I am by no means certain whether all granular magma stains in this way with hematoxylin, and what I have just stated may apply only to a portion of the granular magma.

Specimen No. 531 (shown in figure 8, plate 1) has its cœlom filled with a liquid mass in which there is a granular deposit that surrounds the embryo anlage. Such specimens are numerous and, without opening them, they may frequently be recognized by the transparency of the chorionic wall, which is covered with but few atrophic villi. A more advanced embryo, showing the same condition, is shown in specimen No. 512. In it the embryo is atrophic and macerated, without the presence of an amnion. The chorion is thin and is fully covered with delicate degenerated villi. Other specimens which come within this group are Nos. 21, 78, 122, and 244*a*. These are all illustrated in my monograph on monsters.

Sometimes the entire specimen is filled with a gelatinous mass, which becomes firmer when fixed in formalin and separates into a more solid mass, and into a liquid when preserved in formalin. This mass appears to lie within the amnion in most specimens, as in cases where it fills the whole ovum the amnion is missing. Specimen No. 604 (plate 1, figures 1 and 2) is quite typical, as is also No. 135. In both the embryos are atrophic and necrotic, and the jelly-like fluid fell out with ease when the ovum was cut open. The chorion is atrophic in both of them and is covered only with a few atrophic villi. Specimen No. 604 came to me without a history, and measures 70 by 50 by 50 mm. It is fully covered with fibrinous clots, between which there are few large villi, as the picture shows. The chorionic wall is 3 to 4 mm. in thickness, and its interior is entirely filled with jelly-like magma of uniform consistency. On one side of the specimen, lying free within the hyaline magma, is a straight embryo, 17 mm. in length, with atrophic head, arms, and legs. The same description applies equally well to No. 135. Specimens like these are quite numerous in our collection of human ova, but usually the jelly is lost when the specimen is opened. Figures illustrating embryos of this sort may be seen in my paper on monsters, under the description of embryos Nos. 79, 94, 230, 261, and 270.

No. 1117 (plate 1, figure 5) contains an embryo well packed in the jelly-like magma. The cavity of the ovum is small and its wall is very hemorrhagic. The specimen came from a woman, age 26 years, who was married at 15. She had two births at term and one previous abortion. She believed she became pregnant about 3 months before the operation, although she had not missed her regular periods.

Another specimen belonging to this category is No. 813. It consists of a fleshy mole, well filled with tough, jelly-like magma. All the villi are destroyed and its surface shows ulceration. Further study of this magma is necessary before it can be related to the granular magma which forms with the reticular magma in the exocœlom. I am inclined to believe that the hyaline substance which is so often found within the amnion of pathological specimens arises from the amniotic liquid, which has become richer in albumen, and therefore congeals into a jelly-like mass when preserved in formalin.

CONCLUSION.

The fibrils forming reticular magma are always in direct continuity with those of the mesenchyme of the chorionic wall. This can easily be demonstrated by means of Van Gieson stain, and reticular magma must therefore be viewed as embryonic connective tissue extending into the cavity of the ovum. The stronger strands are accompanied more or less by mesenchyme nuclei, showing that the magma itself must be viewed as independent connective tissue identical with the mesenchyme of the chorion. As the amnion extends these strands are pushed aside, their final remnants being seen in that portion of the exocœlom which encircles the umbilical cord.

In pathological specimens the reticular magma increases in quantity in the earlier stages of development, continuing for a number of months of pregnancy. Frequently the meshes between the reticular fibrils are filled with peculiar stratified granules which take on an extensive hematoxylin stain. Often the amnion is

destroyed early in development, in which case the magma may dissolve, but sometimes it increases greatly in quantity, forming a gelatinous mass. Frequently pathological ova are encountered in which the development of the embryo is retarded, and the amnion is often found filled with a flaky deposit that, as time goes on, increases greatly in quantity and finally forms large crusts which invest the embryo. In other cases there is marked hydramnios, and in certain instances, where the amnion is destroyed, the magma dissolves, leaving only the embryo floating in the fluid encircled by the chorionic wall. Specimens are also found in which the cavity of the amnion is greatly enlarged and is filled with a jelly-like substance, which in later stages may form crusts encircling the embryo. The true relation between the pathological changes of the contents of the exocœlom and of the cavity of the amnion remains to be determined.

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EXPLANATION OF PLATES.

PLATE 1.

FIGS. 1, 2. Photographs of the two halves of ovum No. 601. Natural size. The cavity of the ovum is filled with a jelly-like substance in which a pathological embryo is embedded.

FIG. 3. Section through a normal ovum No. 836, encapsulated in the decidua. $\times 3\frac{1}{2}$. Drawn by Mr. Didusch. The embryo lies within the ectoderm, and bands of magma fibrils radiate from the amnion to the chorionic wall. The head of the embryo shines through the more transparent portion of the amnion.

FIG. 4. Photograph of a block of the ovum, No. 836, *in situ* after the embryo had been removed. $\times 2\frac{1}{2}$. The supporting strands of magma are strikingly shown.

FIG. 5. Pathological embryo No. 1117, embedded in hyaline magma. $\times 1$. From a tubal pregnancy following gonorrhea (?).

FIG. 6. Pathological ovum No. 560, containing a great quantity of reticular magma. $\times 2\frac{1}{2}$. The embryo is normal in form. From a case of retroversion of the uterus.

FIG. 7. Pathological ovum No. 991, with the cavity completely filled with reticular magma. Natural size. The embryo is normal in form. From a negro woman. Sections of the embryo indicate that it is macerated.

FIG. 8. Pathological ovum No. 531, containing a granular deposit around a nodular embryo. $\times 1\frac{1}{2}$.

FIG. 9. Pathological ovum with a nodular embryo (651*g*). $\times 2$. The exocœlom is gorged with granular magma.

FIG. 10. Specimen No. 636. $\times 2\frac{1}{2}$. The embryo and chorion are normal in form, but the reticular magma is markedly increased in quantity.

PLATE 2.

FIG. 1. Pathological embryo No. 512, lying free within the ovum. $\times 6$. The villi are thin and scattered and the embryo is atrophic. There is no formed magma.

FIG. 2. An ovum, No. 576, obtained from tubal pregnancy, showing a delicate layer of magma fibrils around the attachment of the umbilical cord to the chorion. $\times 3$.

FIG. 3. Ovum No. 533, showing very extensive magma. $\times 6$.

PLATE 3.

FIG. 1. Ovum No. 515. $\times 7$. There is a delicate network of fibrils below the amnion and the chorion.

FIG. 2. Embryo No. 588. $\times 8$. Delicate strands are shown radiating from the umbilical cord and yolk-sac. This figure is given to show the appearance of magma in vesicle development. From a woman who has had numerous mechanical abortions performed upon herself. Uterus badly inflamed.

FIG. 3. Section through the chorion and magma of No. 402. $\times 280$. The specimen was stained with Van Gieson stain and shows that the fibrils of the magma are continuous with those of the mesenchyme of the chorionic wall. It came from a case with subinvolution and symptoms of endometritis.

FIG. 4. Outline of the ovum of No. 660. Natural size. The diagram indicates the part of the specimen shown enlarged in figure 5.

FIG. 5. No. 660, showing very extensive changes in the magma. $\times 6$. The upper tip of the amnion is shown. The magma is fibrillar and granular, and at places the fibrils seem to form membranes. The chorionic wall is very hemorrhagic.

Fig. 1 (604)

Fig. 2 (604)

Fig. 3 (836)

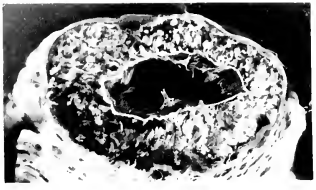


Fig. 4 (836)

Fig. 5 (1117)

Fig. 6 (560)

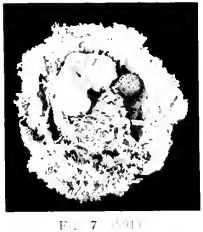


Fig. 7 (561)

Fig. 8 (831)

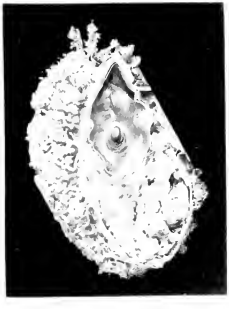


Fig. 9 (857 G)

Fig. 10 (1117)

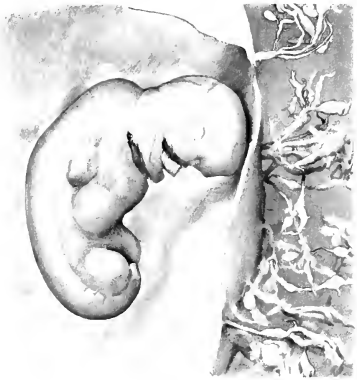
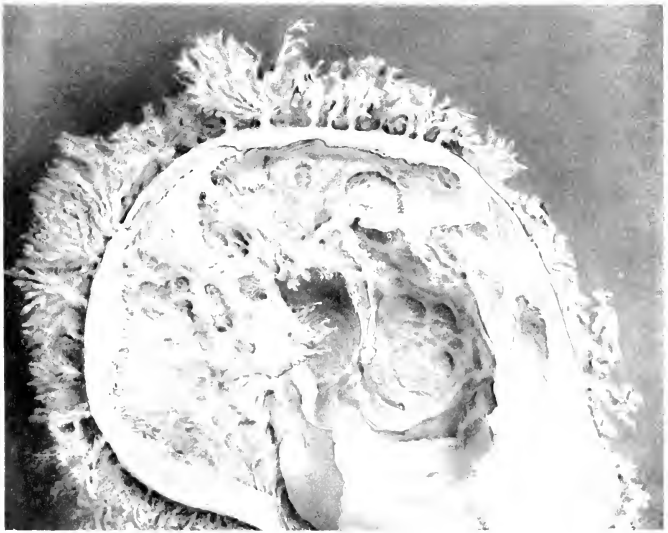


Fig. 1 (512)

Fig. 2 (576)



Didusch fec

Fig. 3 (555)

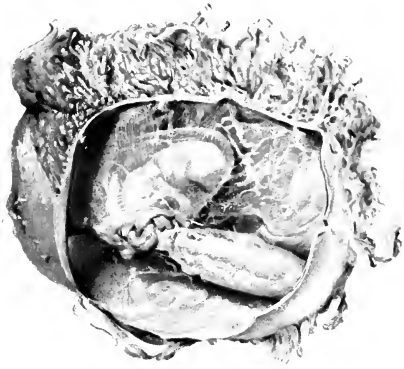


Fig. 1 (545)

Fig. 2 (538)

Fig. 4 (660)

Fig. 3 (302)

Collapsed amnion.

Fig. 5 (660)

Didusch fec

CONTRIBUTIONS TO EMBRYOLOGY, No. 11.

THE STRUCTURE OF CHROMOPHILE CELLS OF THE NERVOUS SYSTEM.

By E. V. Cowdry.

Anatomical Laboratory, Johns Hopkins University.

With one plate.

THE STRUCTURE OF CHROMOPHILE CELLS OF THE NERVOUS SYSTEM.[1]

By E. V. Cowdry.

INTRODUCTION.

It has long been known that certain peculiar nerve-cells, well characterized by their structural appearance, occur in the normal human brain, and indeed in the brains of all the vertebrates which have been examined. In fixed preparations they are slightly shrunken, they stain deeply with both acid and basic dyes, and their nuclei are obscure and hard to define. Flesch (1887, p. 196) called them "chromophile" cells. Nissl (1896, p. 1154) thought at first that they were artefacts of some sort, but Cajal (1909, p. 210) and others brought forward strong evidence against this view. Cajal (1909, p. 211) concluded that they were resting cells. On the other hand, in the light of Dolley's (1910, p. 333) work, they would seem to be in the initial stages of fatigue, as evidenced by the increase in the amount of Nissl substance in them and by their obscure, deeply-staining nuclei. Our knowledge of their structure is incomplete so far as the mitochondria and the canalicular apparatus are concerned. Busacca Archimede (1913, p. 332), alone, has observed that the mitochondria in certain cells in the brain of *Testudo graca* stain particularly intensely with iron hematoxylin, and in some cases seem to lose their definite outlines and to form homogeneous masses. Rina Monti (1915, p. 39) has made a comprehensive study of the canalicular apparatus ("apparati di Golgi") in nerve-cells, but she does not mention cells in the chromophilic condition. I shall consequently venture to present in this paper my observations on these two structures in the chromophile cells in the brain of the white mouse.

MATERIAL AND METHODS.

White mice were employed because they are the smallest mammals which can be conveniently used in the laboratory for experimental purposes. The small size of their nervous system permits the study of the distribution and the arrangement of chromophile cells in serial sections. All the mice were of known age and care was taken that they were perfectly normal.

A modification of the methods of Altmann (1890, p. 27), Galeotti (1895, p. 466), Regaud (1910, p. 296), Bensley (1911, p. 309), and Shirokogoroff (1913, p. 523) was devised for the study of mitochondria. The method has many advantages. In the first place, the use of a mixture of formalin and potassium bichromate as a fixative (Regaud) gives a much more uniform preservation of mitochondria than the osmic acid containing fixatives in general use. The application of the fixative by

[1]The work was aided by the Department of Embryology of the Carnegie Institution of Washington, and part of it was done at the Marine Biological Laboratory, Woods Hole, Massachusetts, where, through the kindness of the Director Dr. Lillie, a room was placed at my disposal.

injection through the blood-vessels (Shirokogoroff) eliminates many very objectionable artefacts due to faulty penetration. The use of permanganate and oxalic acid (Bensley) facilitates the staining of the mitochondria with the anilin fuchsin (Altmann), and the counterstaining with methyl green (Galeotti) permits of the demonstration of the Nissl substance in the same cell with the mitochondria. The fact that the method gives good results in the case of other tissues where the mixtures of Altmann, Flemming, and others are useless on account of their poor powers of penetration, justifies the following detailed statement:

Fixation:

Chloroform the animal. Inject warmed 0.85 per cent NaCl solution into the aorta through the ventricle. If the brain alone is to be studied clamp the descending aorta. If the entire nervous system is to be fixed, clamp the coeliac, the renals, the superior and inferior mesenterics, the iliacs, and the brachials. Continue the injection until the salt solution is returned uncolored through the jugulars. During this time lay bare the arch of the aorta and the carotids from connective tissue, so that they may expand easily and carry more fluid to the brain. Gravity pressure of not more than 6 feet may be employed. Cut the inferior vena cava and the jugulars so that the salt solution may run through easily.

Follow the salt solution with the formalin and bichromate mixture: 3 per cent potassium bichromate, 4 parts; neutral formalin, 1 part. The potassium bichromate acts best when freshly prepared. Neutral formalin is made from the commercial variety by the addition of magnesium carbonate, a deposit of which should always remain at the bottom of the formalin bottle. It is important that the pressure should be at the maximum when the mixture is first injected, so that the blood-vessels may be fixed in a state of dilation. If the pressure is low when the fixative comes in contact with the vessel-walls they will be fixed in a condition of collapse. It will then be difficult, or even impossible, to obtain a complete injection. The injection should be continued for about an hour.

The brain is then dissected out and immersed in the fluid. In the case of the mouse's brain it is sufficient to divide it longitudinally into halves. The fixative must be changed every day for 4 or 5 days, otherwise it undergoes a change evidenced by a darkening in color. This change is accelerated by light and by heat, so that the tissue should be kept in the dark and in a cool place. Fixation may also be effected by simple immersion of the tissue in the fixative, instead of by injection, but this procedure is not recommended.

After this prolonged fixation the tissue is mordanted in a fresh 3 per cent solution of potassium bichromate, in which it remains for 8 or 9 days, changing every second day.

Wash in running water for 24 hours. The object of this careful washing is to remove most of the formalin and bichromate, for otherwise the tissue will be extremely brittle and hard to cut.

Dehydration and embedding:

50 per cent alcohol 12 hours; 70 per cent and 95 per cent alcohol 24 hours each; absolute alcohol 6 to 12 hours; half absolute and xylol 6 hours; xylol 3 hours; paraffin 60° C. 3 hours; cut in 4μ serial sections.

Staining

(1) Pass the sections, mounted on slides, down through toluol, absolute, 95, 70, and 50 per cent alcohol to distilled water.

(2) 1 per cent aqueous solution of potassium permanganate 30 seconds; but this time must be determined experimentally.

(3) 5 per cent aqueous solution of oxalic acid also about 30 seconds.

(4) Rinse in several changes of distilled water about a minute. Incomplete washing prevents the staining with fuchsin.

(5) Stain in Altmann's anilin fuchsin, which is to be made up as follows: Make a saturated solution of anilin oil in distilled water by shaking the two together (anilin oil goes into solution in water in about 1 per cent.). Filter and add 20 grams of acid fuchsin to 100 c.c. of the filtrate. The stain should be ready to use in about 24 hours. It goes bad in about a month. To stain, dry the slide with a towel, except the small area to which the sections are attached. Cover the sections on the slide with a small amount of the stain and heat over a spirit lamp until fumes, smelling strongly of anilin oil,

come off. Allow to cool. Let the stain remain on the sections for about 6 minutes. Return the stain to the bottle.

(6) Dry off most of the stain with a towel and rinse in distilled water, so that the only stain remaining is in the sections. If a large amount of the free stain remains it will form a troublesome precipitate with the methyl green; on the other hand, if too much stain is removed the coloration of the mitochondria will be impaired.

(7) Again dry the slide with a towel, except for the area covered by sections. Allow a little 1 per cent methyl green, added with a pipette, to flow over the sections, holding the slide over a piece of white paper so that the colors may be seen. Apply the methyl green for about 5 seconds at first and then modify the time to suit the needs of the tissue.

(8) Drain off excess of stain and plunge the slide into 95 per cent alcohol for a second or two, then rinse in absolute for the same time, clear in toluol, and mount in balsam.

Several difficulties may be met with: (1) The methyl green may remove all the fuchsin, even when it is only applied for a short time. This is due to incomplete mordanting of the mitochondria by the chrome salts in the fixative. It may often be avoided, either by omitting the treatment with permanganate and oxalic acid, or by treating the sections with a 2 per cent solution of potassium bichromate for a few minutes immediately before staining (as advised by Bensley). The action of the permanganate and oxalic is to remove the excess of bichromate. (2) Or the fuchsin may stain so intensely that the methyl green removes it very slowly or not at all. This, on the other hand, is due to too much mordanting. It may be corrected by prolonging the action of the permanganate and oxalic. (3) Sometimes, after obtaining a good differentiation, the methyl green is washed out before the slide is placed in toluol. This may be avoided by omitting the 95 per cent alcohol, by passing from the methyl green to the absolute direct. (4) Unfortunately the stain is not very permanent. Under favorable conditions it will last for 3 or 4 years. The fading in color is hastened by light and by heat, and it proceeds very rapidly in a damp atmosphere.

Cajal's (1912, p. 211) uranium-nitrate method was employed for the canalicular apparatus in its original form, except for the substitution of methyl green in the place of carmalum as a counterstain.

Control preparations were fixed in a variety of fluids and were stained in many ways, as will appear later.

The figures have been made from specimens prepared by the above-mentioned fuchsin-methyl green method, by which the mitochondria are stained red, the Nissl substance green, while the canalicular apparatus remains uncolored; and also from specimens made by the uranium-nitrate method, which blackens the canalicular apparatus and colors the Nissl substance green.

OBSERVATIONS.

Chromophile cells, as the name implies, possess an unusual affinity for stains, which may be either acid or basic. Their structure is variable. A glance at the figures is sufficient to show this. The variations may represent stages in a process, which, when pushed to an extreme, results in a cell in an advanced stage of chromophilia, but of this we have no conclusive proof. Neither can we assert that the process proceeds in this direction, for the changes observed may equally well be interpreted as taking place in the reverse order. We do not yet know whether the series is homogeneous; that to say, whether we are not arbitrarily grouping several processes of different nature under the same heading. For instance, a mitochondrial increase (figures 1 and 2) may not precede a diffuse staining of the whole cell with mitochondrial dyes (figure 6), which may be brought about in an entirely different way. Nevertheless, the cells are all chromophile in the sense already defined, that they stain deeply.

Some chromophile cells differ only from other cells by a slight increase in the amount and in the intensity of the staining of the mitochondria (figure 1). There is apparently no corresponding change in the Nissl substance and the morphology of the mitochondria is unaltered.

Other cells show a remarkable increase in the number of mitochondria. For example, a cell (figure 2) frequently contains three or four times as many mitochondria as its neighbor; this increase in mitochondria is associated with a slight but perceptible increase in the amount of the diffuse Nissl substance in the cytoplasm and with a darker staining of the acidophilic and basophilic nucleoli and the ground-substance of the nucleus. Cells in this condition show no evidence of shrinkage. They may be recognized in Cajal preparations (figure 7) by the changes in the nucleus and the Nissl substance. The Cajal preparations show that the canalicular apparatus is unaltered.

There may be a great increase in the Nissl substance, which is present as a diffuse deposit (figure 3). At the same time some of the mitochondria often lose their discrete outlines and seem to merge into the surrounding cytoplasm. Mitochondria may not be very numerous in cells of this kind. The nucleus stains intensely and a few clear canals may be seen in its vicinity. The cell has apparently shrinkage spaces on either side of it. Preparations, made by fixing in alcohol and staining with toluidin blue, contain cells in which the Nissl substance is in this condition and Cajal preparations show that the canals are unaltered.

Figure 4 illustrates a cell in a rather more advanced stage of chromophilia. In this cell there is an unusually large amount of Nissl substance and there are further evidences of the disappearance of formed mitochondria, especially in the cell process. The outlines of the nucleus can barely be made out. The canalicular apparatus shows no modifications either by this method or by the Cajal technique.

A very interesting condition is shown in figure 5. Here, with this degree of differentiation, only a few typical mitochondria persist near the origin of the cell process. The Nissl substance is overshadowed by a cloud of material staining the same way as the formed mitochondria do in adjacent cells. Figure 8 illustrates a similar cell in a Cajal preparation. The Nissl substance in it is increased and there is no modification in the blackened canalicular apparatus. Cells in this condition are often shrunken. It is difficult to determine whether the shrinkage is the expression of an actual diminution in the size of the cells during life, or whether it is simply the result of a difference in the reaction of chromophile cells to the fixation and subsequent treatment. The presence of what appear to be shrinkage spaces around the cells seems to indicate that it is in reality due to the technique employed, because if, on the other hand, it was due to a decrease in the size of the cell during life, one would expect the space to be filled up by a shifting of neighboring structures. It may be emphasized that the fact that other cells, in actual contact with chromophile cells, show no signs whatever of shrinkage must be regarded as one of the distinctive properties of cells in the chromophilic condition. There is, of course, still another interpretation, namely, that the spaces in question are unusually large perineuronal spaces, the enlargement being in some way connected with the difference in the physiological condition of chromophile cells as contrasted with other cells.

The mitochondria may apparently disappear more or less completely in certain cells, and their place be taken by a mass of amorphous material with the same staining properties (figure 6). The nucleus may or may not be visible. Cajal preparations of cells in the same condition (figure 9) show that the canals are unaltered. The nucleus is obscured by the cloud of Nissl substance. The appearance of these cells, in advanced stages of chromophilia, would perhaps lead one to suppose that they are degenerating and that their nuclei have disappeared. That this is not the case may be seen if one of the mitochondrial preparations is stained with hematoxylin and eosin. The hematoxylin and eosin does not color either the amorphous deposit or the Nissl substance, which, in the mitochondrial and in the Cajal preparations, hides the nuclei. The nuclei have in reality distinct and definite outlines and appear to be quite unaltered, except that they contain rather more than the usual amount of chromatin. In fact, the change in the mitochondria and the increase in the amount of the Nissl substance would never have been suspected if hematoxylin and eosin had alone been used.

The distribution of chromophile cells is important. They often occur singly. They may be surrounded on all sides by cells which show no tendency toward an assumption of the chromophilic condition. They may, on the other hand, occur in clumps. The clumps vary greatly in size. They contain cells in all stages of chromophilia in addition to a variable number of unaltered cells, which are always present, scattered among them.

The neuropil in which the chromophile cells are embedded differs in no way from the neuropil elsewhere. It seems, by all the mitochondrial methods, to be studded with mitochondria. But it must not be thought that the mitochondria occur in anything like equal numbers in the neuropil of different regions, because there is a remarkable variation in this respect. The mitochondria appear to be intercellular, but unhappily a source of error is introduced by the fact that the unmedullated, and to a lesser extent the medullated, processes stain in much the same way as the mitochondria, so that in some cases it is impossible to distinguish between them. Undoubtedly a large number of the mitochondria in the neuropil are contained in nerve-cell processes cut in section, but there is no *a priori* reason why they should not occur free from the cells as an intercellular deposit. This important question can only be solved by a detailed study of staining reactions, possibly by the elaboration of new methods, or by taking advantage of the differential solubilities of mitochondria. It has a direct bearing upon the rôle of intercellular material in the metabolism of the central nervous system.

Cells in the chromophilic condition are comparatively rare in the olfactory bulb as compared with the cerebral cortex. In fact, they are more abundant in the cerebral cortex than in any other part of the brain. Clumps of them are more common here than in other regions. The clumps vary in size, in shape, and in position in the brains of animals from the same litter, apparently treated in exactly the same way. Chromophile cells are also numerous in the hippocampus. They are, on the contrary, comparatively rare in the corpus striatum and in the thalamus, in both of which they are more frequently met with singly than in groups. In the midbrain they are found in about the same number. It is interesting to note that they are

quite numerous in the cerebellar cortex. The Purkinje cells are particularly liable to show this condition. They are infrequent in the medulla and they scarcely ever occur in the spinal cord, in the spinal ganglia, or in the sensory ganglia of the cranial nerves, as, for example, the Gasserian ganglion. In other words, this remarkable condition of the nerve-cell is more prevalent in the higher centers than in the lower ones. This is particularly true of chromophile cells in advanced stages of chromophilia.

The question at once arises as to whether these changes in the appearance of the cells are indicative of real alterations in the cells themselves or whether they are merely the result of the treatment to which they have been subjected.

Unfortunately it was found impossible to confirm these observations by the study of unstained, living cells by reason of the difficulties met with in attempts to isolate the cells without injuring them. Attempts to stain the mitochondria in living cells by injecting a solution of janus green into the brain through the blood-vessels did not yield satisfactory results because the janus green was almost immediately reduced, first to the leucobase, and then to the red diethylsafranin, by the reducing action of the brain-substance and the absence of an adequate supply of atmospheric oxygen, so that observations could not be made. Pure oxygen was bubbled through the janus-green solution while it was being injected, in the hope that the reduction of the janus green might thus be retarded, but without success. Attempts to tease out individual cells in the nervous system and to stain them by simple immersion in the janus-green solution resulted, of course, in a coloration of the mitochondria, but it was on the whole unsatisfactory on account of the unavoidable injury to the cells. Consequently I have had to rely solely upon the study of fixed material.

The results obtained with the fuchsin-methyl green method and with the Cajal technique have been confirmed by the detailed examination of material stained by the Benda method, the Altmann method, and with iron hematoxylin. Chromophile cells are, I think, not artefacts due to alcohol fixation, as Barker (1899, p. 124) supposes, because I have observed them in tissues fixed in a great variety of fluids not containing alcohol. Moreover, Flesch (1887, p. 197) found years ago that they could be identified in the fresh, unstained condition as well as in tissues stained vitally with methylene blue.

The fact that the chromophile cells are very abundant in the superficial layers of the cortex would at first seem to indicate, as some investigators believe, that they are artefacts due to mechanical manipulation. The clusters of chromophile cells are sometimes cone-shaped, with the base on the surface of the cortex and the apex of the cone extending inwards, which looks as if they might have been produced by pressure from without which radiated inwards. But isolated clumps of chromophile cells occur in deeper parts of the brain, which can not be explained in this way. Moreover, a number of other facts seem to be incompatible with this view. In the first place, since all the brains were fixed, before removal, by the injection of the fixative through the blood-vessels, it follows that there could be no mechanical injury until after fixation. The invariable occurrence of unaltered cells, side by side with the chromophile cells, is hard to explain on the basis of mechanical injury, because whatever pressure had been brought to bear upon the tissue must necessarily

have acted upon both; but one shows the condition and the other does not (as is shown in all the figures). Furthermore, if mechanical injury is the cause of the condition, it is difficult to understand why chromophile cells are so rare in the spinal cord and in the ganglia of the cranial nerves, which are bound down by membranes and which in removal are consequently subjected to greater mechanical injury than the cortex of the brain.

In order to settle the question the results of intentional mechanical injury brought about by bruising the cerebrum and the spinal ganglia with a blunt instrument were studied. It was found that the lesion produced was characterized by the flattening or compression of many cells in the same direction, at right angles to the direction in which the pressure had been exerted. All the cells in the area were uniformly affected. Normal cells were not scattered among them. The injured cells stained intensely, but they did not simulate the chromophile cells. The neuropil between them showed marked changes and could readily be distinguished from the neuropil elsewhere in the same section.

Chromophile cells are not the result of differences in the time or in the degree of fixation. The whole brain is uniformly fixed by the methods of technique employed. The distribution of chromophile cells is not related to the arrangement of the blood-vessels, which are the avenues of approach of the fixative. Neither do the mitochondria vary in number with the vascularity of the region.

The condition is not due to irregular mordanting with the potassium bichromate, because complete extraction of the bichromate by prolonged treatment with permanganate and oxalic acid does not essentially modify the appearance of the chromophile cells when the sections are stained.

Another possibility is that the intense staining of the chromophile cells results from incomplete differentiation. Even if this were the case the differences in the rate of decolorization must be the visible expression of real differences in the cells themselves. I have found, however, that the same differences obtain in undifferentiated specimens stained lightly with fuchsin, crystal violet, and iron hematoxylin. I have made a number of experiments to determine whether more complete differentiation would bring to light formed mitochondria in cells in which they appear to have been replaced by the amorphous deposit which stains in the same way.

Specimens were stained in the usual fashion with fuchsin and methyl green and were mounted in balsam. Drawings were then made of chromophile cells which had been stained intensely with the fuchsin and in which no formed mitochondria could be seen. The cover-glass was then dissolved off and the slide was passed down through toluol and graded alcohols to water. It was then restained with fuchsin, differentiated more strongly with the methyl green, mounted in balsam, and examined. The same condition was apparent, except that the homogeneous deposit had a distinctly greenish color. The same process was repeated as many as five times with the same cell, increasing each time the extent of differentiation, until the cell stained intensely with methyl green and very little trace of the fuchsin was left; still no formed mitochondria were observed; this was repeated with other cells with the result that in some of them formed mitochondria were brought to light, while in others they were not.

Similar experiments were performed with individual cells stained a homogeneous black color with iron hematoxylin. The results obtained are easier to interpret because the differentiator, iron alum, does not itself color the tissue like the methyl green. This advantage is counterbalanced by the fact that both the mitochondria and the Nissl substance stain in the same way and it is often difficult to distinguish between them. In many cases, particularly in slightly undifferentiated specimens, the extraction of the stain from chromophile cells by further differentiation brought to light a variable number of formed mitochondria. Moreover, it is worthy of note that the chromophile cells in the cerebral cortex are the last to become decolorized and that the differentiation occurs with unequal rapidity in different parts of the cell, thus indicating that the homogeneous deposit is not present in the same concentration in all parts of the cell.

The end-result of this experimentation is that chromophile cells, particularly those in advanced stages of the condition, contain a diffuse deposit, which stains in a typical way with all mitochondrial dyes, and which is probably formed by the solution of some of the mitochondria in the cell.

The condition is not due to technique and it is not associated with a visible pathological change on the part of the animal.

All the mice employed were apparently normal. They ate well and showed no signs of sickness. They were killed with chloroform, and it may at once be said that the changes are not due to acute chloroform poisoning, because animals killed in other ways, by decapitation, for example, showed the same condition. The mice were not excited, or disturbed or exercised in any unusual way before they were killed. A careful autopsy of each mouse was made to make sure that it was quite normal. Some were found to contain a parasite, present in the cysticercus stage in the liver; these were invariably discarded. The chromophile cells were found in mice of both sexes in almost all seasons of the year. They were found in mice varying in age from 25 days to adults, so that they can not be regarded as an expression of senility. It was thought that they might occur in consequence of abnormal conditions due to captivity. In order to settle this point a wild field-mouse was captured alive and in good condition and its brain was prepared in the usual way. It, also, showed chromophile cells.

An apparently analogous partial solution of mitochondria was observed in liver-cells poisoned with phosphorus by Mayer, Rathery, and Schaeffer (1914, p. 609). Accordingly, W. J. M. Scott tried the effect of experimental phosphorus poisoning on the nervous system of white mice. The chromophile cells were apparently entirely unaffected and a solution of mitochondria was not brought about. Dr. Bensley made the interesting suggestion to me that this partial solution of mitochondria in chromophile cells might be due to a swing of the reaction in them toward the acid side, with the liberation of free fatty acids. I therefore made some preliminary experiments on acidosis in mice produced by the subcutaneous injection of dilute hydrochloric acid, all of which yielded negative results as far as the chromophile cells were concerned. I have, further, found that slight exercise does not alter the appearance of the chromophile cells in the brains of white mice to any noticeable extent.

It seems highly probable, therefore, that chromophile cells occur normally in the brain of the white mouse and that we have to reckon with a partial solution of mitochondria just as we have for many years recognized a chromatolysis, or solution of the Nissl substance.

DISCUSSION.

This work on chromophile cells has, I believe, an important bearing upon (1) the question of differential nerve-cell activity; (2) the phenomena of chondriolysis and hyperchromatism; (3) the functional independence of the mitochondria and the canalicular apparatus; and (4) our conception of the structure of living nerve-cells.

(1) The distribution of chromophile cells in the different parts of the brain is interesting. The fact that they occur most abundantly in the cerebral cortex and in the cerebellum, and that they are rarely found in the lower centers like the spinal cord, would seem to indicate that the central neurones differ in some way from the more peripheral ones. The difference may be one of lability, for Dolley (1914, p. 56) has found that more highly specialized cells are more prone than less specialized ones to respond with structural changes to physiological experimentation. Moreover, the occurrence of these cells in groups, which vary in size and in position in different brains, is in accordance with our conception of the alternation of rest and activity in the higher centers and may well have some bearing upon the vexed problem of cortical localization, for as yet neither the mitochondria nor the canalicular apparatus have been considered in this connection.

(2) We must recognize a "chondriolysis," or a partial solution of mitochondria, in nerve-cells as well as a "chromatolysis." The word "chondriolysis" was first employed by Romeis (1912, p. 139) to describe the disintegration of certain mitochondria which escaped from the cells into the uterine fluid of *Ascaris*. It is, to my mind, more appropriate than the term "chromatolysis," which is frequently applied to the so-called solution of Nissl bodies, for the simple reason that I am of the opinion (1914, p. 20) that the Nissl substance is usually in solution in the living nerve-cell, whereas the mitochondria are assuredly present as definite formed bodies (except of course in the chromophilic condition).

Chemical changes are undoubtedly involved in the phenomena of conduction (Tashiro and Adams, 1914, p. 329) and, in view of the distinct differences in the chemical constitution of the mitochondria and of the Nissl substance, the one being of a lipoid albumin nature (Fauré-Fremiet, Mayer and Schaeffer 1910, p. 95) and the other being apparently a complex nucleoprotein containing iron (Scott, 1905, p. 507), it seems probable that the study of mitochondria and the changes which they undergo may bring to light variations in the activity of the nerve-cell which could never be detected by the study of the Nissl substance alone. Quite apart from the rôle of the nucleus in the elaboration of the Nissl substance and the purely cytoplasmic nature of mitochondria, there is further evidence of a functional diversity between the two structures. I have found that in the nerve-cells of the mouse the mitochondria vary directly with the volume of the cytoplasm and that the Nissl substance varies inversely with the nucleus cytoplasmic ratio; also that the mitochondria are of more general occurrence in nerve-cells than the Nissl substance.

They are present in the granule-cells of the cerebellum, as is also evident from the earlier work of Altmann (1890, plate XIII, figure 1) and Nageotte (1909, p. 826), and in the granule-cells of the olfactory bulb of mice and rats, which are well known to be devoid of Nissl substance. Moreover, in certain cell-groups, under normal conditions, there is often a variation in the mitochondria, as between different cells, without any corresponding change in the Nissl substance. Mitochondria occur abundantly throughout the length of the axone, where no Nissl substance has ever been seen. They also occur in certain dendritic processes which do not contain any Nissl substance. Evidence of this sort may be multiplied.

Just how the mitochondria are concerned with the activity of the nervous system is unknown. I have presented evidence elsewhere (1914, p. 18) that they play a part in the basic processes of metabolism which are common to all cells, but this is unfortunately a very broad statement and we naturally desire to learn something rather more specific about them. Coghill's (1915, p. 350) belief that the mitochondria are concerned in the constructive (anabolic) side of metabolism is of interest in this connection, particularly since it falls so well in line with the well-known "eclectosome" theory of Regaud (1911, p. 699), which, in turn, is an extension of the "side chain" theory of Ehrlich. M. R. and W. H. Lewis (1915, p. 393) make the interesting suggestion that the mitochondria take part in cellular respiration, which is also a fundamental process common to all cells.

We may confidently expect that this new avenue of approach to the study of the activity of the nervous system will yield results of importance, not only because our histological methods of technique are now sufficiently accurate to permit of the actual enumeration of the mitochondria, a thing which can not be accomplished in the case of the Nissl substance, but also because Waldemar and Mathilde Koch (1913, p. 427) have recently succeeded in devising chemical methods for the qualitative and quantitative estimation of substances, very closely related, perhaps identical with mitochondria, in the nervous system. These substances are phospholipins. Hoppe Seyler long ago pointed out that lecithin (a typical phospholipin) and cholesterol are to be found almost everywhere that life phenomena exist. In fact, a great wave of revived interest is manifested in recent chemical and pathological literature in these complex compounds of fatty acid, phosphorus, and nitrogen. Mathews (1915, p. 88) very aptly remarks that the phospholipins are the most important substances in living matter:

> "For they are found in all cells, and it is undoubtedly their function to produce, with cholesterol, the peculiar semifluid, semisolid state of protoplasm. The latter holds much water in it, but it does not dissolve. Indeed it may be said that the phospholipins with cholesterol make the essential substratum of living matter. This physical substratum of phospholipin differs in different cells and probably in the same type of cell in different animals, but everywhere, from the lowest plants to the highly differentiated brain cells of mammals and of man himself, it possesses certain fundamental chemical and physical properties. In all cases the phospholipin substratum is soluble in alcohol containing some water," etc.

In view of these considerations it is interesting to inquire whether the distribution of mitochondria in cells corresponds with that of the phospholipins. It is certainly true that mitochondria are more widely distributed than any other kind

of cell granulation now known to us. They occur in almost all cells. Yet certain cells, like the fully differentiated non-nucleated red blood-cell, unquestionably contain a large amount of phospholipin, though no formed mitochondria can be seen. The mitochondrial substance is probably present in solution, just as it appears to be in chromophile cells, for it would obviously be absurd to state that it must always occur in that state of condensation which makes it visible with the aid of certain powers of the microscope. The recent investigations of Levene (1915, p. 41) on cephalin are of interest. A new field of investigation is evidently opened up. It may thus be possible to pursue this line of work with chemical as well as with histological and physiological methods, a combination which has been but rarely effected.

Work along these lines seems the more desirable since, as will be seen, it may throw new light upon certain problems in the pathological anatomy of the nervous system as well. Wells (1907, p. 460), in his discussion of mental fatigue, writes:

> "Since the lecithin forms so important a part of the nervous system, it is tempting to imagine that in fatigue excessive quantities of its toxic decomposition product, *cholin*, and the still more toxic derivative of cholin, *neurin*, are formed in considerable amounts and cause part, at least, of the intoxication."

Now we have seen that, in the opinion of certain investigators, mitochondria are largely composed of lecithin. It is possible, therefore, if Wells's reasoning is correct, that the symptoms of mental fatigue are the result of their decomposition. Moreover, Halliburton (1907, p. 74) and others are convinced that organic diseases of the nervous system may be distinguished from functional neuroses on account of the formation of cholin in the one and not in the other. This opens up the possibility of a differentiation between these two great groups of diseases on the basis of cell structure, as to whether or not there is a change in the mitochondria.

(3) The persistence of the canalicular apparatus in chromophile cells is of interest in general cytology. In chromophile cells, in which there are marked structural changes, the canalicular apparatus remains without any great modification. This is rather surprising, since investigators have gradually come to regard the canalicular apparatus as the most labile cell organ; but it is in conformity with Key's as yet unpublished observations on degenerative changes in spinal ganglion cells. Key finds that the canalicular apparatus persists without much modification for from 12 to 24 hours after death in spinal-ganglion cells left in the animal.

I have shown (1912, p. 494) that a canalicular apparatus, in the form of a system of clear, uncolored canals, occurs in the same cell with typical mitochondria and that consequently the canalicular apparatus and the mitochondria are structurally distinct. This conclusion is strongly supported by my observation that they may likewise be seen together in chromophile cells, the difference being that while the mitochondria are greatly changed, the canalicular apparatus remains with little or no modification, so that they are functionally as well as structurally different. My positive impregnations of the canalicular apparatus by the uranium-nitrate method of Cajal confirm this observation.

Now, Cajal (1908, p. 123) is so certain of the identity of the clear canals (described originally by Holmgren) and the "Apparato reticolare interno" of Golgi

that he refers to them as "conduits de Golgi-Holmgren." But Rina Monti (1915, p. 40) has made the statement that the large internal reticular apparatus corresponds to the chondriome (*i. e.*, to mitochondria) in the nerve-cells of mammals; to quote her own words: "Il grande apparato reticolare interno dal Golgi nelle cellule nervose di mammiferi corrisponde adunque al condrioma, comme il grande apparato descritto dal Pensa nelle cellule cartilaginea." If Cajal is correct in his identification, it would appear that the canalicular apparatus and the mitochondria are identical. I have already discussed (1912, p. 490) the older statements of Popoff (1906, p. 258), Smirnow (1906, p. 153), Van Durme (1907, p. 84), Meves (1908, p. 846), and Hoven (1910, p. 479), who are inclined to believe this to be the case.

It is hard to see how these two views can be reconciled. I am inclined to think that the well-known lack of specificity of the methods of silver impregnation which Pensa (1913, p. 560) and Rina Monti (1915, p. 45) have employed are the cause of the confusion. I do not believe that the Golgi method can be trusted invariably to demonstrate a certain structure within the cell, like the canalicular apparatus; and, for this reason, I can not accept unreservedly Cajal's identification of the canalicular apparatus with the Golgi apparatus. I agree with Duesberg that a more precise definition of the "Apparato reticolare interno" is highly desirable, but I do not agree with him in his attempt (1914, p. 37) to define it in terms of its relation to the centrosome, because our knowledge of the centrosome itself is so deplorably inadequate. We require, above all else, more accurate methods before the matter can be cleared up.

(4) This discussion of the structure of chromophile cells may be profitably concluded by a statement of our present knowledge of the cytoplasmic structure of living nerve-cells of vertebrates not in the chromophilic condition. Mitochondria unquestionably occur and may be seen as such in living nerve-cells even without any vital stain. The Nissl substance is usually present in solution, not in the form of discrete masses as seen in fixed preparations. I believe that there is also an amorphous argentophilic material which (when treated by appropriate but very capricious methods) assumes the form of fibrils. The canalicular apparatus, like the neurofibrils, is an unknown quantity in living nerve-cells, although it may be demonstrated in fixed tissues with considerable regularity. These structures, or more correctly speaking substances, are distinct and should not be confused with one another. Although the mitochondria alone have a definite morphology and can usually be seen in living nerve-cells, under ordinary conditions, with the present means at our disposal, it would be arbitrary in the extreme to say that the others can never be seen. Pigment, fat, lipoid, etc., may of course be seen in variable amount in living nerve-cells. It is the more fundamental constituents with which we are concerned.

The recent work in bio-chemistry, summarized by F. Gowland Hopkins (1913, p. 663) in his presidential address before the Physiological Section of the British Association, has, I believe, an important bearing here. The cell is regarded as a dynamic system of co-existing phases in more or less stable equilibrium, the condition of which is altered, from moment to moment, by processes of oxidation, reduction, desaturation, condensation, etc., which naturally result in physical changes in the cell, with the building-up and breaking-down of molecular aggregates which may or

may not be visible with the microscope or the ultra-microscope. The Nissl substance, argentophilous material, etc., doubtless undergo changes of this sort from liquid to fluid and semi-solid phases. It seems right and proper, therefore, to steer an intermediate course, as I have done, between those, on the one hand, who assert that the Nissl substance and the neuro-fibrils occur in living cells in approximately the same form as they appear in fixed and stained preparations, and those, on the other hand, who claim that they are artefacts pure and simple and that they can never be seen in the living cell. Our problem is more one of material than it is of form.

In this connection the solution of mitochondria in chromophile cells is a phenomenon of considerable significance. In addition to variations in the chemical constitution of mitochondria, there is also evidence to the effect that there may be variations in the condensation or density of the mitochondrial substance (*vide* Duesberg, 1915, p. 40). This is a factor which has been too often ignored. We are inclined to look for mitochondria in all cells which are functionally active and in which metabolic changes are taking place. The fluidity of the mitochondrial substance varies and I am prepared to believe that further investigation will bring to light cells which are active functionally, but in which no trace of formed mitochondria may be seen.

CONCLUSIONS.

(1) Chromophile cells occur under normal conditions in the brains of white mice.

(2) They are distributed unequally in the different parts of the nervous system. They are most abundant in the cerebral cortex. They are progressively less abundant in the cerebellum, corpus striatum, thalamus, midbrain, and medulla. They are of very rare occurrence in the spinal cord, spinal ganglia, and sensory ganglia of the cranial nerves.

(3) This restriction of the chromophile cells to the higher centers is in full accord with the well-known lability of the central, more highly specialized cells as contrasted with the more primitive, peripheral neurones.

(4) Chromophile cells, as seen in fixed and stained preparations, vary greatly in structure. There is usually more or less shrinkage of the cell-body. The nucleus may also be shrunken. The acidophilic and basophilic nucleoli are particularly prominent and the ground-substance of the nucleus stains intensely with both acid and basic dyes. There is an increase in the amount of Nissl substance. The Nissl bodies become confluent and form a homogeneous mass. The cell is hyperchromatic. The canalicular apparatus is unaltered. The mitochondria either increase in number and stain more intensely, or else some of them lose their discrete outlines and form a diffuse deposit which stains intensely by the mitochondrial methods of technique. This change in the mitochondria occurs in the cell processes in the neighborhood of the cell, as well as in the cell-body. Although the nucleus may be completely obscured by this cloud of mitochondrial substance, it still remains and stains in the usual way with hematoxylin and eosin.

(5) The lability of the mitochondria and the constancy of the canalicular apparatus in chromophile cells confirms my earlier contention by showing that the two structures are physiologically as well as anatomically distinct.

EXPLANATION OF FIGURES.

All the figures have been drawn with Zeiss apochromatic objective 1.5 mm. compensating ocular 6 and camera lucida giving a magnification of 1,500 diameters. The figures have not been reduced in reproduction. In all of them unaltered cells are represented side by side with chromophile cells just as they occur in the preparations.

Figures 1 to 6 represent cells in the cerebral cortex of a male white mouse, 26 days old and weighing 5 grams. The brain was cut into serial sections 4 μ in thickness and stained with fuchsin and methyl green. All the figures were drawn from cells in the same section to insure uniformity in the action of the stain and of the differentiator. The mitochondria are stained red, the Nissl substance green, and the canalicular apparatus persists, in some of the cells, in the form of clear, uncolored spaces.

Figures 7 to 9 represent cells from the cerebral cortex of a male white mouse, 29 days old and weighing 10 grams. Portions of the brain were prepared by the uranium-nitrate method of Cajal and were cut into serial sections 1 μ thick. These figures were also drawn from a single section to insure uniformity in the action of the counterstain, methyl green. The canalicular apparatus is in the form of a blackened network and the Nissl substance is colored green.

FIG. 1. Two cells, having a distinct increase in amount and intensity of the staining of the mitochondria. This change may mark the first stages in the assumption of the chromophilic condition.

2. A much greater increase in amount of mitochondria and a slight increase in intensity of the staining of the Nissl substance and the nucleus.

3. The Nissl substance is more abundant. It is diffuse and stains more brightly. The outlines of the mitochondria are indistinct. The nucleus stains darkly. A few clear canals are visible near it. There is what appears to be a shrinkage space on either side of the cell.

4. Still greater changes. The mitochondria appear to be going into solution; outlines of nucleus barely distinguishable.

5. The mitochondria have almost all gone into solution. The Nissl substance is almost entirely obscured by the cloud of mitochondrial material which stains with the most energetic of the two dyes, acid fuchsin. The nucleus is invisible.

6. A complete "chondriolysis" or solution of the mitochondria. The canalicular apparatus is present in the vicinity of the nucleus.

7. The increase in amount of the Nissl substance indicates a slight degree of chromophilia. The canalicular apparatus is blackened and shows no changes.

8. Greater increase in the Nissl substance. It is diffuse, with marked hyperchromatism. The nucleus stains diffusely with methyl green. Its outlines are obscure. The canalicular apparatus, in black, is unaltered and the cell as a whole is shrunken.

9. Cell so intensely stained with the methyl green that the nucleus can not be seen. Canalicular apparatus slightly condensed, otherwise unchanged. There is a considerable shrinkage of the cell.

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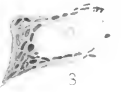

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CONTRIBUTIONS TO EMBRYOLOGY, No. 12.

ON THE DEVELOPMENT OF THE LYMPHATICS OF THE LUNGS IN THE EMBRYO PIG.

By R. S. Cunningham.

With five plates.

CONTENTS.

ON THE DEVELOPMENT OF THE LYMPHATICS OF THE LUNGS IN THE EMBRYO PIG.

By R. S. Cunningham.

From an analysis of the literature on the development of the lymphatic system, it is clear that there is a general agreement among recent workers that the mammalian lymph-sacs precede the lymph-vessels in the time of their appearance, and hence constitute what may be called a primary lymphatic system. This system consists, in mammals, of 8 sacs: 3 paired, the jugular, the subclavian, and the posterior iliac lymph-sacs; and 2 unpaired, the retroperitoneal sac and the cysterna chyli.

The further development of the lymphatic system—that is, the formation of the thoracic ducts and the peripheral vessels—has been discussed at length by numerous workers during the past decade. These workers have been grouped into two general schools: the one holding that the lymphatics grow by a centrifugal sprouting of pre-existing endothelium, the other believing that these vessels are formed by a coalescence of numerous isolated spaces developing in the mesenchyme.

According to the centrifugal theory, briefly stated, the sacs arise from the veins and are joined together by vessels that sprout out from their endothelial walls. Thus the thoracic duct arises from both the retroperitoneal sac and the left jugular sac, and the two elements unite somewhere between the two points of origin. Supporters of the centrifugal theory claim that the secondary lymphatic system (the capillary bed) arises by the sprouting of the endothelial walls of the sacs and of the right and left thoracic ducts. These sprouts invade the organs and, becoming progressively more complex, assume the adult form of the lymphatic system. The supporters of the multiple-anlagen theories (whether they believe in coalescing tissue-spaces, multiple venous origins, or degenerating veno-lymphatics) agree in claiming that lymphatics do not grow by the centrifugal sprouting of the pre-existing endothelial walls.

It is not my intention to review here all the various theories that have been advanced, but only to call attention to the two general views, in order to correlate my findings with them. A very thorough discussion of these two views, as well as a comprehensive review of the literature, may be found in the Ergebnisse der Anatomie und Entwickelungsgeschichte, 1913. (Dr. F. R. Sabin, Der Ursprung und die Entwickelung des Lymphgefässsystems.)

Though primarily concerned with the problems of origin and the method of growth of the lymphatic vessels, the supporters of both theories have aided in establishing the morphology of the primary system and have laid the foundation for the further study of the development of the system as a whole. If the centrifugal

theory is correct, it is clear that it should be possible to follow the growth of lymphatics from the sacs into any organ or group of organs. It should also be possible to demonstrate in progressively older stages constantly increasing lymphatic zones and decreasing non-lymphatic zones. The development of the lymphatics of the skin, of the intestine, and of the lung has now been studied in this manner.

In 1904, Dr. F. R. Sabin demonstrated that the skin received its lymphatic supply from the two jugular sacs and the two iliac sacs. From each of these sacs a group of radiating vessels invade the skin and form there a close-meshed plexus. These four plexuses gradually increase in size and finally unite, so that the entire skin is supplied with lymphatics. The differentiation which takes place varies with the location and depends upon the adaptation which the vessels must make to the other structures. Continuing the work of Baetjer (1908) on the retroperitoneal sac, Heuer (1909) studied the development of the intestinal lymphatics by the injection of this sac. He observed and described progressive changes in the intestinal supply, finding more complex injections possible in each older stage. He interpreted these results to mean that the lymphatics had not extended beyond the point which his injections reached and that the region beyond this point constituted a non-lymphatic zone.

There is, therefore, a primary and a secondary lymphatic system. The former consists of a series of sacs formed from the veins and connected by the right and left thoracic ducts. The secondary system consists of the peripheral vessels, which are held by some to be outgrowths from the sacs and by others to be formed *in situ*. With regard to the development of these peripheral vessels, only those of the skin and the intestine had been studied. There was need, therefore, for the study of the other abdominal and the thoracic lymphatics. This work was begun to establish a clearer conception of the development of the secondary system.

In presenting this study, I do not claim to have found any new evidence as to the mode of growth of lymphatics. This work supports the centrifugal theory in the same manner as does that of Heuer (1909); and it is certain that the theory is sufficiently well established to serve as a basis for this work. It is the object of the present paper to follow the gross morphological changes in the development of the lymphatic vessels of the lung from the primary stage to the adult form. It is desired to indicate the general lines of growth and the various stages which the system passes through in the course of its development. No attempt has been made to study the finer structure of the vessels or the mode of growth.

It is important to note that complete injections are very difficult to make, and that it is also difficult to be certain whether the injection in a particular specimen is complete or not. Therefore it is not claimed that any of the injections are complete; and the limits of the lymphatic and the non-lymphatic zones at any stage are defined in a general manner, depending on the comparison of a number of specimens.

The lymphatic supply of the lungs develops from three sources: the thoracic duct, the right thoracic duct, and the cephalad portion of the retroperitoneal sac. In 1913, Sabin remarked: "The right lymphatic duct curves ventralward and grows to the heart and lungs." This is the only statement which I have been able to find

in the literature regarding the development of the cardiac and pulmonary lymphatics from the right duct, or the morphological fate of the right duct in mammals. In the same report attention was called to the fact that the lung-vessels can be injected from the retroperitoneal sac, but this was not studied further at that time. The right duct grows primarily to the heart, just as the left grows to the aorta, this asymmetry depending upon that of the cardio-vascular system, according to the general rule that the principal lymphatic trunks follow the large blood-vessels, and grow with the greatest rapidity where the blood-supply is most abundant.

In the beginning I wish to lay emphasis upon the fact that the lung lymphatics develop partly from the retroperitoneal sac, and to call attention to the fact that these vessels persist in the adult as part of the permanent drainage of the lung, and hence may be of importance in pathology. On account of the complexity of the development of the lung lymphatics, it has seemed best to present this work, not by describing and figuring a series of progressively more complex specimens, but by describing the development as a consecutive growth and illustrating with those preparations that may seem best to clarify the text. However, as a matter of reference, the following table of periods has been arranged, in order to offer a brief outline of the complexity at varying stages. These stages are selected with regard to the more important principles of growth and are as follows:

(1) The downgrowth of the two ducts, completion of the primitive system, and the first vessels to the trachea and lungs. Embryos 2.3 to 3.5 cm.

(2) The migration of the heart; the coalescence of the cardiac drainage with that of the lungs, by the formation of the tracheal plexus and the plexus on the arch of the aorta; the growth of the vessels in the lung from the earliest sprouts along the bronchi to the primitive pleural plexus, and the early marking-off of the connective-tissue septa; and the growth up from the retroperitoneal sac through the ligamentum latum and the anastomosis in the primitive septa into which the vessels grow. Embryos 3.5 to 4.5 cm.

(3) The completion of the primary lymphatic system; that is, when the entire organ is supplied, and the further development is in an increasing complexity of the plexuses already present, incident to the increase in the size of the organ and its assumption of mature activities. During this period the formation of the valves and nodes begins. Embryos 4.5 to 7 cm.

(4) The remainder of the development is considered a period, as it is, in reality, an adaptation of the system already present to the increasing needs of the organ. This includes the differentiation of the drainage-lines and the final development of the nodes.

In describing the development of the lymphatics of the lung, the growth of the left duct down to the aorta, of the right duct to the heart, and the formation of the primitive tracheal plexus and the early vessels to the lungs from both ducts will be considered first; the further development of the tracheal plexus, together with the changes incident to the descent of the heart, will follow; then the origin of the vessels from the retroperitoneal sac and their growth up through the ligamentum pulmonale into the lungs will be considered. After the anastomoses of the two sets of lymphatics, the lung will be considered as a whole, inasmuch as the further development is symmetrical for the entire organ, with the exception of the final lines of drainage and the development of the nodes.

I wish to express here my indebtedness to Professor F. R. Sabin for her constant advice and assistance throughout this work. Also I wish to thank Mr. James F. Didusch and Miss Flora Schaeffer for the illustrations.

METHODS.

The injection method has been principally used, but it has been supplemented and supported by evidence from both single and serial sections. The collection of pig embryo of the Anatomical Laboratory has been at my disposal, and I have also studied a number of especially prepared series. Many of the series have been of embryos in which the blood-vessels have been injected, and this has materially aided in their interpretation; in fact, in all the especially prepared series the blood-vessels were injected. All these embryos were fixed in Carnoy's fixing fluid, consisting of 6 parts of absolute alcohol, 3 parts of chloroform, and 1 part of glacial acetic acid.

The method of fixation is as follows: Place the embryo immediately in the fluid and allow it to remain there 6 to 8 hours; then transfer directly to 70 per cent alcohol; dehydrate by ascending grades of alcohol with 2 per cent difference until 95 per cent is reached; then change to absolute. This gives excellent fixation with very little shrinkage. The stains used were Ehrlich's hematoxylin and a mixture of eosin, aurantia, and orange G.

The injection masses used were india ink, a saturated solution of prussian blue, a 5 per cent aqueous solution of silver nitrate, and an aqueous suspension of lampblack. The india ink and prussian blue give about the same results, except that the specimens injected with prussian blue are more easily studied after clearing, as the ink renders them more opaque. The india ink, however, flows more easily and hence the injections are more nearly complete. The silver-nitrate injections are easiest to analyze and give beautiful preparations, but its caustic action prevents the finer vessels from filling, so that only the larger trunks are injected; however, it furnishes an extremely valuable method of following the principal drainage-lines at different stages. The lampblack is the mass which gives the most nearly complete injections, but unfortunately it precipitates in fine flakes and gives a feathery appearance to the specimen, thus rendering it difficult to use for illustrating.

It will be necessary to review the methods used in injecting the various stages, as they differ considerably and are of especial importance in interpreting the results. The earliest injections were made by filling the jugular sacs from the superficial plexuses and then gently moving the embryo. I have succeeded in injecting the early vessels to the trachea and the lungs in only a few pigs less than 3 cm. long, because the injection mass usually follows the path of least resistance, which is into the jugular vein.

In injecting embryos between 3 and 6 cm. in length, three general methods have been employed:

(1) The best and by far the easiest method of obtaining good preparations of the left part of the tracheal plexus is to inject through the retroperitoneal sac in the manner described by Heuer (1909); but this seldom gives good preparations of any of the vessels of the lung except those of the lower lobe. However, this method has been of particular importance in following the lymphatics up from the retroperitoneal sac to the posterior poles of the lower lobes.

(2) One may inject the tracheal plexus, especially the left part, by plunging the needle deep behind the aorta and injecting cerebralwards; the right plexus is sometimes filled also, and often the vessels of the left lobe of the lung.

(3) Finally, the vessels of the lung are best injected by a puncture just ventral to the trachea (the tracheal plexus) and behind the arch of the aorta. Here the tracheal plexus is always extravasated, but the lung-vessels fill up nicely.

The embryos older than these mentioned, that is, longer than 7 cm. (or after the valves are formed), are much more difficult to inject, and this difficulty increases with further development. The method employed has been to inject directly into the connective-tissue septa of the lung and to continue the injection slowly until there is some extravasation at the point of puncture, when a part of the lung surrounding the area of extravasation is well injected. This method has been very satisfactory in all specimens that were obtained very soon after the removal of the uterus; most of the injections were made while the heart was still beating.

In order to study the relations between the blood-vessels, bronchi, and lymphatics, multiple injections had to be made. Various combinations were employed. In some, the lymphatics were injected together with veins and arteries; in others with either veins or arteries alone. Again, the lymphatics and the bronchi were injected; and in still others the lymphatics were combined with either veins or arteries. In these multiple injections prussian blue, india ink, and carmine were used, the lymphatics being injected with either the blue or the ink.

The specimens in which three systems were injected were difficult to clear, unless only the large bronchi and blood-vessels were filled.

In order to trace the vessels more accurately, many of the injected lungs were embedded in paraffin and cut in thick serial sections (100 to 500μ); these were mounted in balsam but not stained. Other lungs were cut at 10 to 20μ and stained similarly to the series already referred to.

All measurements of embryos refer to crown-rump diameter and were taken before fixation, as is customary in this laboratory. The illustrations are labeled "C. R. —"; this refers to the crown-rump measurement.

In 1906, Flint published his study on the development of the lungs in the pig, and his work has been taken as a basis of the general structure of the lungs, especially with reference to the development of the bronchi and blood-vessels. He reviewed all the important literature on the embryology of the mammalian lung, studied the lymphatics in sections, and briefly summarized their structure and distribution at various stages, but he did not attempt to inject them. I have been able to confirm most of his observations. However, he labored under the difficulty of having neither reconstructions nor injections. He gives a short summary of each stage, and of these summaries I quote the more important parts:

Stage 3 cm.: At the root of the lung a few dilated lymphatics may be noted near the bronchi and pulmonary vessels; however, they have not grown beyond this point into the substance of the lung wings.

Stage 5 cm.: From the root of the lung the lymphatics have gone some distance into its substance. They have thin walls composed of young fibrils lined with endothelium with occasional valves. They are confined, however, to the immediate neighborhood of the main bronchi and their chief subdivisions.

Stage 7 cm.: The most interesting change, however, lies in the further growth of the lymphatics, which in the earlier stages are found in the root of the lung in the neighborhood of the pulmonary vessels and the large bronchi. As they grow in, they accompany these structures for a distance;

then approaching the end branches they leave them and run in a plexiform manner midway between the bronchial tubes until they reach the pleura. This gives the lung now an indefinitely lobulated appearance in which the periphery of the simple lobule is indicated by the lymphatic vessels and the center by the bronchi. The lymphatics are lined with flattened endothelium; their walls are formed by the young connective-tissue fibrils, and here and there valves are beautifully shown which, in general, point away from the pleura.

Stage 13 cm.: The lymphatics, forming a plexus around the bronchial veins and arteries at the root of the lung, accompany them towards the periphery, giving off branches to the interlobular spaces en route. * * * On reaching the periphery of the lung they leave these structures and pass out, as in the preceding stages, to the pleura. They have a plexiform arrangement and may be traced at times into the substance of the lobules. This course may be observed in the deeper lobules of the lung as well as in those on the surface under the pleura.

Stage 19 cm.: In general the relations of the lymphatic system have not changed.

Stage 23 cm.: At 23 cm. the first evidence of the submucous lymphatic system is seen in the stem bronchi. It may, however, be found earlier, but the vessels are difficult to follow. It would seem thus that we have in the pig's lung, besides the lymphatic plexuses accompanying the bronchi, arteries, and veins, an interlobular system which Miller has been unable to find in the human lung. Injections pointing to such a relationship he has interpreted as artefacts. If Miller's conclusions prove correct, then the lymphatics of the human lung must develop, so far as the interlobular system is concerned, in some other way.

I quote at length from Flint because he alone, of the many workers on lung lymphatics, has approached the subject from the embryological side. As I have said, Flint was seriously handicapped by having only sections from which to draw his conclusions. He was especially struck by the prominence of the vessels lying in the interlobular septa, and attempted to explain their apparent change of course (*i. e.*, from the bronchi to the septa) by the theory that the density of the tissue was greater around the bronchi and vessels and that the lymphatics chose the path of least resistance. He did not call attention to the relation of the veins to this point in the development of the lymphatics, which will be discussed later, but emphasized the fact, so amply shown by injections, that these interlobular vessels grow much more rapidly than the vessels around the bronchi and arteries.

It will be necessary hereafter to discuss the work of Miller on the adult lymphatic system, in connection with the later stages; therefore it will suffice to refer here to the statement which Flint discussed in the quotation given above. Miller has called attention to the fact that the terminal vein lies in the periphery of the lobule and that the lymphatics accompanying the vein communicate with those of the pleura. He cites Councilman's (1900) description of the interlobular vessels, but does not claim to have found the same vessels. I think that these different views will be reconcilable when we have followed the development of the lymphatics through the various stages that lead to the adult form. The literature on the lymphatics of the adult mammalian lung is very large, and for a comprehensive review of it the reader is referred to the papers of Miller (1893, 1896, 1900, 1902, 1911). It seems needless to discuss it more at length here.

THE VESSELS ARISING FROM THE LEFT DUCT.

As has been said, the lymphatics of the lungs arise partly from the two thoracic ducts by sprouts. These vessels grow to the mesenchymal wall of the trachea and form there a plexus which sends vessels down into the lungs. Other vessels grow directly into the lungs.

The thoracic duct, as has been shown by Sabin (1913), Baetjer (1908), and Kampmeier (1912), is complete—that is, it connects the jugular sac with the retroperitoneal sac—in a pig embryo 2.5 cm. long. Very soon after this the first evidence of the pulmonary supply may be found. I have obtained partial injections at 2.8 cm., and have found some small vessels in serial sections at 2.6 cm.; so it is evident that these sprouts are either formed from the thoracic duct as it grows down or very soon after the primary system is completed.

About midway between the jugular anastomosis and the arch of the aorta the thoracic duct leaves its position lateral to the trachea and bends dorsalward to lie near the dorso-lateral border of the esophagus. In this position it comes down behind the arch of the aorta. This transition is shown by Sabin (1913, figures 12 and 13). Just at the point where the duct begins to bend dorsally the earliest sprout to the lung is formed. At this point a single large vessel buds off from the thoracic duct and passes down over the arch of the aorta to reach the hilum of the lung. This vessel unites with the vessels that grow up from the thoracic duct just caudal to the arch of the aorta and forms the lower part of the tracheal plexus. This vessel usually persists in the adult as one of the drainage trunks from the hilar nodes to the thoracic duct. It is shown in figure 5, plate 1, and figure 2, plate 4, marked with an asterisk. From the region of the thoracic duct, where this vessel buds off to a point about the level of the aortic arch, a number of other vessels are formed very soon afterwards. These vessels arise very close together and grow across to the lateral wall of the trachea, where they anastomose and form the primitive left tracheal plexus; they lie in the undifferentiated mesenchymal tissue that surrounds the tracheal lumen. These lymphatics have formed a plexus by the time the embryo has reached a length of 3 cm. From this plexus vessels grow across the trachea to anastomose with other vessels from the similar plexus on the opposite side; other lymphatics grow up the trachea and form a coarse-meshed plexus around it. This is the anlage of the adult supply of that structure. But the most important of the branches of this plexus, as far as the present work is concerned, are those from the lower part. These pass down the trachea and, being joined by other vessels that leave the duct near the arch, pass up over the bifurcation and into the lung. The left tracheal plexus is shown in figure 5, plate 1, and figures 1 and 3, plate 2. Here must be noted the fact that the plexus of the left side supplies the greater portion of the ventral surface of the trachea and forms the largest part of the great sheet of lymphatics around the primary bronchi. Later these vessels anastomose freely with those from the right side. It is important to call especial attention to the difference in the richness of the supply of the dorsal and the ventral surfaces of the trachea. There are vessels that grow to each from the left plexus, but a much greater number pass to the ventral surface than to the dorsal. Thus the plexus formed from the two lateral groups is much more closely meshed on the ventral surface, and from it is derived the greater part of the lung supply. Over the bifurcation there is a very complex group of vessels, and these form tubes around the principal bronchi as they grow on into the lung.

Below the level of the hilum several vessels, three or four in number, grow up from the thoracic duct and its plexus surrounding the aorta, to join with the large

vessel which has been described as the first to the lung and which comes over the arch to reach the hilum. These vessels from the duct below the hilum form a plexus with the vessel from above, as has been described. It is well known that the thoracic duct is double below the level of the arch of the aorta and that the two divisions are connected by numerous anastomostic vessels (figure 1, plate 2). This system is the anlage of the vessels that surround the aorta in the adult. This relation has been figured by Heuer (1909). One of the lymphatics that pass up from below to join the first vessel from the thoracic duct above leaves the duct near the diaphragm and is consequently very conspicuous in injections of this region. Heuer has figured this lymphatic as one that goes to the heart, a conclusion entirely justifiable from the general appearance of the injected specimen. Figure 1, plate 2, is from a dissected embryo 4 cm. long, in which the lymphatics were injected from the retroperitoneal sac. The thoracic duct and part of the left tracheal plexus are injected, and the extension of the plexus down on the bronchus is also shown. Below the arch may be seen some of the vessels that grow up to meet the branch from above. These vessels have been cut off, with the arch, to expose the tracheal plexus. The double duct is also shown, the more ventral element being the one figured by Heuer.

The pulmonary vessels reach the hilum when the embryo is about 2.8 cm. long, and can be seen in sections at 3 cm. (see figure 1, plate 1). The lung-tissue is at this time very slightly differentiated mesenchyme, containing the early bronchi and blood-vessels. For a further description of the structure of the lung at this stage see Flint (1906). These early lymphatics are grouped in an irregular manner in the hilum of the lung and may be found at 2.9 and 3 cm. in sections. But I have not been able to inject them earlier than 3.3 and 3.5 cm. Figure 1, plate 1, is of a section from an embryo 3 cm. long, in which the blood-vessels were injected while the embryo was still living. The lymphatics are shown as a few dilated spaces (blue) in the hilum. These vessels are beginning their invasion of the lung-tissue while the tracheal plexus is forming. It is necessary, however, to complete the description of this plexus before considering the portion of this study which relates to the lung proper. The development of the vessels within the lung-substance will be considered after the formation of the right lymphatic plexus has been described. It is important, however, to note here that all the vessels to the left lung come from the closely united group of vessels on the trachea and around the aortic arch, as has been described. This will be studied in relation to the first vessels to the lung on the right side, which will next be considered.

On the right side the development is, in general, similar to that on the left, but differs in a few particulars, chiefly relating to and in consequence of the asymmetry of the vascular system. The right duct is primarily to the heart, or perhaps to the vena cava, since it follows that vessel to reach the cardiac base. But while the heart supply is at first only from the right side, the vessels to the lung and the trachea develop at about the same time. The right duct grows caudalward parallel to the thoracic duct to the point where the vena cava arches ventralward to reach the heart. There it divides, and one branch follows the posterior wall of the vena cava to reach the cardiac base, while the other passes into the hilum of the lung. The

cardiac division, after reaching the base of the heart, along the posterior wall of the vena cava, passes around the bulbous arteriosus to reach the anterior surface of the heart, where it divides to form the primitive pericardial plexus. By introducing a canula dorsal to the vena cava and injecting towards the heart, I was able to fill this plexus in a pig 3 cm. long. At this stage it extends about one-fourth of the distance from the base to the apex of the heart. Figure 13 in Volume V of the Johns Hopkins Hospital Reports, Monograph Series (Sabin on "The Origin and Development of the Lymphatic System"), shows the right duct near the heart in an embryo pig 2.5 cm. long. In that paper attention was called to the fact that the duct grows towards the heart and that it probably represents the cardiac supply.

The second of the two terminal branches of the right duct passes down parallel to the dorsal wall of the trachea in about the same general position as that occupied by the duct above the point of division. Thus it might seem proper to consider the lung division as the more fundamental of the two, as it appears to be the continuation of the undivided duct. However, the heart branch is probably the more fundamental and the earlier of the two, since it is a general principle in the growth of lymphatic trunks for the principal vessels to follow the larger blood vascular channels. Hence we consider the left duct as primarily aortic and the right as primarily cardiac in distribution.

This vessel enters the hilum of the lung and breaks up into a few branches that are grouped around the bronchi and blood-vessels as on the left. The nature of the grouping and the further development are similar on the two sides, and hence both will be considered together. There is, however, an interesting difference between the two upper lobes, which is dependent upon the relation of the aortic arch to the hilum on the left. On the right the lung is distinctly higher (*i. e.*, nearer the neck) than on the left, because on the latter side the aortic arch lies in the groove made at the juncture of the upper lobe with the trachea. Thus the vein to the upper lobe on the left passes close to the bronchus under the aortic arch, while on the right it is well above the bronchus. This allows more freedom in the lymphatic growth on the right, so that the vessels to the upper lobe come down directly into it instead of growing back from a single group, as they do on the left. It must be understood that the stage referred to is between 2.5 and 3 cm., when the heart is still higher than the bifurcation. Later the heart passes still farther down into the thoracic cavity, and these differences disappear as the cardiac and aortic relations to the lung begin to assume their adult form. There is, however, one very important effect of this asymmetry; the lymphatics of the right duct pass directly into the lung, while those of the left must course up over the arch of the aorta and the bifurcation of the trachea to reach the lung-tissue. This has been mentioned briefly before. It is clear that the principal supply of the bronchi, and therefore, ultimately, of the lungs, comes from the left duct. This is in large measure the result of the asymmetric relations of the heart and aorta.

The development of the first vessels to the trachea and lungs on the right side will next be described in detail. From the heart limb of the right duct a few vessels arise and grow down over the vein to the upper lobe on the right side; after crossing the vein they enter the lung near the hilum and divide into several branches, some

of which anastomose with those mentioned above as growing down into the hilum of the lung from the pulmonary limb of the right duct. Other vessels turn outwards along the bronchi and veins and grow into the lung-tissue of the upper lobe. This process will be described later.

Along the right duct, cephalad to the division into the two branches, other vessels are given off; some grow down to anastomose with ascending branches lying along the tracheal wall and coming from the vessels described above, while others grow to the tracheal wall at varying positions along the section lying between the jugular anastomosis and the bifurcation, corresponding somewhat to the vessels on the other side, with which their branches anastomose, forming the tracheal supply. The earliest injection of the lymphatics of the right side were at 2.8 and 2.9 cm.

Figure 2, plate 3, shows an embryo of 3 cm., where the injection was made into the right sac, which illustrates the relative position of the vessels to the upper right lobe and the limb that follows the vena cava to the heart. This drawing is diagrammatic and does not show the different vessels to the lobes on the right side, though some of them were injected. The left duct is shown without any branches.

In figure 1, plate 2, the right tracheal plexus is represented. Though it is very incomplete, it shows the general form of the plexus and its relation to the similar plexus on the other side. The right tracheal plexus, in its simplest form, consists of a few vessels which are beginning to anastomose along the lateral wall. These anastomoses become more and more complex and numerous until, along the right side of the trachea, a plexus somewhat similar to that of the other side is formed. They differ, however, in that on the right there is no aortic arch to complicate the form. Therefore the plexus is a simple sheet-like group of vessels which lie along the lateral wall of the trachea, but do not extend up over the ventral surface of the bifurcation, except by a few anastomosing vessels. It anastomoses freely with the larger plexus from the other side on the ventral surface of the trachea, and later the combined plexuses lose their individuality and appear continuous. In the meantime the two tracheal plexuses have begun to anastomose. This will next be described.

Between 3.3 and 4.5 cm. the two tracheal plexuses anastomose by means of numerous vessels which grow around the trachea, both dorsally and ventrally. Above the level of the aortic arch these connecting vessels are far less numerous than below, where the two are merged into a sheet-like plexus that surrounds the trachea and passes down into the lungs as tubes of vessels surrounding the bronchi. Above the bifurcation the dorsal surface of the trachea has fewer vessels than the ventral, while the two original lateral plexuses are much more closely meshed, representing the anlagen of the two lateral groups of lymph nodes of the adult.

From the close-meshed plexus on the left side of the trachea just at the bifurcation a group of lymphatics pass up over the left stem bronchus and sweep across to the right bronchus, forming the upper group of vessels lying on the bronchial wall. These grow down on the side and anastomose with the vessels coming down from the plexus on the right side. Thus it will be seen that the left supply is a more important part of the general origin than the right, supplying, as it does, all of the left lung and part of the right.

It is of importance to note here that the heart is migrating downwards (*i. e.*, caudalwards) during this period, and, by the time the embryo has reached 4.5 cm. in length it has come to lie almost directly over the hilum of the lung. Hence the vessels that formerly ran in a long course from their point of origin in the heart limb of the right thoracic duct to reach the upper lobe and the hilum of the lung have become a part of the common tracheal plexus, and the formerly distinct duct to the heart has also been absorbed by the plexus over the bifurcation.

The cardiac vessels then (at 4.5 cm.) drain directly into the plexus over the hilum of the lung (figures 1 and 3, plate 2). This relation remains in the adult in the drainage of the cardiac vessels into the mediastinal nodes and the union of the efferent trunks of these nodes with those from the hilum of the lungs.

Here must be mentioned, though not bearing particularly on the lymphatics of the lungs, the connection between the right and the left ducts. In specimens of about 3.5 to 4 cm. in length, I have regularly found a vessel arising from the dorsal part of the right tracheal plexus and joining the thoracic duct behind the aorta. As has been said, it seems best to consider the vessel to the heart as the continuation of the right thoracic duct; hence this vessel must be considered, as was the one to the lung, as a part of the collateral supply.

The lung, as has been stated, also derives lymphatics from another source—the cephalad portion of the retroperitoneal sac. These vessels are growing into the lung during the period when those already described are differentiating, but it seems best to postpone the discussion of this portion of the pulmonic supply until we have studied the early changes that take place in the lung itself, following the invasion by the vessels already described. The desirability of this is evident when it is remembered that the vessels from below must follow a similar course in the lung, with the exception that this course is reversed, due to the fact that these vessels invade the lung through the pleura instead of the hilum, and must reach the other supply through the interlobular septa, to be described later.

At 3 cm. there are two primary bronchi and two veins on either side, one of each to each upper lobe and one to each lower lobe. From these the secondary branches are beginning to form. From 3 cm. to 5 cm., these secondary branches are developing rapidly and are very large in comparison to the size of the lung. The arteries are very much smaller, and the veins are somewhat larger than the arteries, but much smaller than the bronchi. It is of great importance to note the relations of these structures to each other during this period. Flint has studied their development very thoroughly, but he does not call attention to the fact, so important with reference to the lymphatics, that the developing vein is separated as widely as possible from the bronchus with which it is morphologically associated. The artery, on the other hand, follows the bronchus very closely and is distributed with it to the center of the developing lobule. The two primary branches of the pulmonary vein lie close to the corresponding bronchi. This is, indeed, as far separate as is possible, since there is almost no lung-tissue at this period, while the secondary vessels which may be considered the terminal branches lie about equidistant from the two adjacent bronchi. The arteries follow the bronchi more closely. This fact is of the greatest importance in the development of the lymphatics and also in the relation of the veins to the periphery of the lobule in the adult, as has been shown by Miller (1900).

As the lung increases in size and the veins and bronchi which we have termed secondary give off other branches, these in turn become the terminal ones and assume the relations that have been described. The others are, by the increasing amount of lung-tissue, forced closer together. Thus it is seen that it is only the terminal veins that occupy the position described; that is, pass along the periphery of the lobule. In the pig there is considerable connective tissue forming definite lobules in the adult lung; and these septa, bounding as they do the area supplied by terminal bronchi, divide the lung into a large number of irregular cones or pyramids, which have the bronchus and artery in the center and the veins passing along the periphery until close to the apex, where they enter veins of the next larger size. For further discussion of this arrangement see Miller's article (1900).

As we have seen, a few dilated lymphatics are found in the hilum of the lung at 2.9 and 3 cm. These are the first branches from the vessels that are forming the plexus on the trachei and bronchi already described. The bronchi, as has been said, are surrounded by lymphatics which follow them into the lung-tissue; and, as secondary bronchi are formed, lymphatics from these plexuses branch off to follow them.

The primary veins lie very close to the corresponding bronchi at this stage, and are accompanied by a few lymphatic trunks which arise from the same general plexus that covers the bifurcation. These vessels anastomose very richly with those of the bronchi, and, close to the point where the trachèa divides, they merge together. We have seen that the secondary veins lie midway between the adjacent bronchi, and represent the outer border of the primitive lobule of the developing lung. Along these veins the lymphatics grow towards the pleura; they are derived both from the plexus that follows the primary vein and from the vessels that surround the primary bronchi. The lymphatics from the bronchial supply join those from the vein, and the combined group passes along the vein, spreading out on either side to form a sheet, until the vessels reach the pleura. Flint observed these sheets of lymphatics, but thought that there must be some difference in the density of the tissues to account for their leaving the bronchi to run midway between. He did not recognize the relation between the veins and the lymphatics. It will be clear, when it is remembered that the smaller branches of one vein spread out fan-like to meet those of the other vein, that the sheets of lymphatics lying between the bronchi are directed by the veins as well as the separate lymph-vessels directly associated with them.

In this manner the true primitive lobules are formed by the interpolation of a sheet of rapidly growing lymphatics between the bronchial tubes. It is along the distal margin of these plexuses that the pleural marking begins. When these vessels reach the pleura there is a marking-out of the characteristic coarsely-meshed plexus, each interspace corresponding to the sheet beneath (figure 3, plate 1). It must be remembered that these vessels, growing as they do very rapidly, reach the pleura very early, and hence the pleural plexus is developing while the above-mentioned interlobular plexuses are forming. We have so far described only the formation of the large parallel plexuses shown in figure 1, plate 4, figure 2, plate 5, and figure 1, plate 3. But the formation of veins in other planes directs the growth of the lym-

phatics, so that with each bronchus there are several veins and several sheets of lymphatics developing. Thus the series of cone-shaped or pyramid-shaped lobules are surrounded by plexuses of lymphatics. Along these plexuses the differentiation of the connective-tissue layers takes place, for, when the lymphatics invade these areas, there is only an undifferentiated tissue, which is characteristic of the lung. Flint suggested that the lymphatics followed the bronchi for a certain distance and then turned away midway between them, because of some relative difference in the density of the tissues. It is quite impossible to observe the relation to the veins in uninjected sections, and consequently this point was not discussed in relation to the problem of the question of tissue density. Notwithstanding this phase of the development which Flint was unable to follow, there still remains considerable probability in his suggestion. The fundamental reason for the direction of growth is as yet entirely a mystery, but there seems to be little doubt that the principal lines of lymphatic development are along the larger blood-channels; and, in general, the veins are chosen, though the left duct may be considered as following the aorta.

The much slower-growing lymph-vessels on the bronchi follow each branch out towards the periphery. The primary bronchus is surrounded by a very close-meshed plexus, which consists of a large number of vessels; in cross-section one can count from 50 to 75. However, this number is very greatly reduced on the secondary bronchi, each of which has four or five trunks following it. These are closely bound together by anastomosing collaterals.

With reference to the secondary bronchi, almost the same series of events occur as given above for the primary ones. These secondary bronchi are likewise marked off by interlobular septa in which the lymphatics develop more rapidly than along the bronchus whose lobule they mark off. The lymphatics around the bronchus give off small vessels near each branch of the bronchus, and these pass across to join the plexuses that surround the area of the lobule (figure 1, plate 3). As the new-formed bronchi grow larger they are, in turn, followed by two or three lymphatics, which end, as did those around the secondary bronchi, by passing over to join the septa or, if close to the pleura, the vessels there. These lymphatics that pass from the bronchial system to join those in the septa follow the branches of the veins which bend in from the septa to reach the capillary bed of the arterial tree. These persist in the adult as the vessels that pass from the bronchus to the vein and thence to the pleura (figure 2, plate 1).

We will consider now the lymphatics that grow up from the retroperitoneal sac into the caudal pole of the lower lobe.

In 1906 F. T. Lewis described, in rabbit embryos, a lymphatic sac just median to the mesonephritic vein. Baetjer (1908) showed that it arises from the ventral surface of the large vein which connects the two Wolffian bodies (embryos 17 to 23 mm.); Heuer, following Baetjer, found that numerous lymphatic sprouts arise from this sac and invade the intestine through the mesentery. This sac supplies lymph-vessels to the stomach, the liver capsule, the Wolffian bodies, and the reproductive glands.

The lower pole of the lower lobe of the lung is continuous with the mesentery in the early stages. As the embryo develops, this connection becomes a thin band

of tissue that passes down behind the diaphragm to end in the tissue around the aorta; it corresponds to the ligamentum pulmonale in the human. It is through this prolongation of the lower lobe that the lymphatics from the retroperitoneal sac grow up to reach the lung. These vessels arise from the cephalad portion of the sac and pass up behind the dorsal wall of the stomach to enter this long posterior or lower pole of the lung (figure 2, plate 4). There are three or four vessels that grow out from the sac and up into the lung; these are closely associated with those that pass to the diaphragm and, in adult life, join with them just before reaching the nodes into which they drain. They pass upward and divide, on reaching the lung, into two groups, one of which passes up over the diaphragmatic surface and the other over the outer or lateral surface of the lower lobe.

The anlage of the ligamentum pulmonale is connected not only with the lower pole of the lung, but also with the median border of the lower lobe. Thus the lymphatics grow directly up about one-third of the way to the hilum in this medial extension of the ligament, and from there sweep out in a fork-like division which produces the two plexuses on the two borders of the lung (figure 3, plate 5). I have injected these vessels at 3.4 cm.; but I think that they reach the lung border a little earlier.

From the two plexuses described above vessels grow into the lung in exactly the reverse order to that followed by those developing from the hilum. They grow in just where they will meet the veins, and along these form the septal plexuses, exactly similar to those described above. These rapidly anastomose with the other lymphatics, and, by the time the embryo has reached 4 cm. in length, the entire lung is uniformly supplied.

It is very pertinent to inquire why the lymphatics that reach the lung from below select these points for the invasion of the deeper tissue of the lung. However, when it is recalled that the lymphatic vessels which lie in the mesenchymal tissue (the pleural anlaga) are very large in proportion to the other structures and that the budding vessel would be in direct relation to the outgoing veins, it is easily understood that exactly the same causes must be acting here as those which direct the growth from above. So here, as above, the position of the veins controls the direction of growth. Of course, the plexuses on the two surfaces become more complex as the lung is invaded and follow the same steps as the pleural supply in general. As has been said, there are branches along the pleura, and these anastomose with the other pleural vessels, so that the supply becomes general. The drainage in the early stages—that is, before the formation of the valves—is probably divided; the flow of lymph might be to the retroperitoneal sac via the vessels that grow up from that structure, or to the thoracic ducts through the tracheal plexuses and the vessels accompanying the veins and the bronchi.

We have seen how the lymphatics grow into the lung-tissue and there form two distinct groups, and how one of these rapidly reaches the pleura and there forms the characteristic plexus-pattern marking off the boundaries of the lobules; also how the vessels grow into the posterior poles of the lower lobes and anastomose with the system from above, which follows the veins in the connective-tissue septa.

Now, it will be well to review briefly the state of the development of the lung lymphatics at the time that the primary system is complete – that is, in 6 cm. embryos. At 6 cm. the lymphatics around the trachea form a close-meshed plexus near the bifurcation, extending down into the lung around the bronchi. Above the bifurcation there are only a few connecting vessels on the ventral and dorsal surfaces of the trachea, but the two plexuses on the lateral surface are very close-meshed. From the left plexus the principal supply of both lungs is derived, but there are numerous vessels passing down into the right lung from the right plexus, and the two are closely bound together, especially near the bifurcation, where they have fused into one plexus. The vessels surrounding the bronchi follow them towards the periphery, giving off branches to the venous tree at every division of the bronchial tree. Each smaller bronchus derives its lymphatic supply from the plexus that accompanies the parent bronchus. These vessels are very difficult to inject.

Accompanying the primary divisions of the pulmonary vein there is another group of vessels that is closely bound, by anastomoses, to the lymphatics around the principal bronchi (figure 4, plate 1). Along each of the tributary veins vessels pass to the pleura and spread out in the region that has been described as the septa between the lobules. Each of these dividing sheets anastomose with other sheets and with the pleural vessels. The vessels derived from the retroperitoneal sac are continuous with those derived from the two ducts; there can be determined no line of differentiation either within the lung-tissue or on the pleural surface. The posterior pole is connected with the retroperitoneal sac by three or four vessels that pass down in the fold of tissue that precedes the ligamentum pulmonale (figure 3, plate 5). The pleural plexus has begun to form within the gross markings that we have described as corresponding to the connective-tissue septa. These vessels are very superficial and are not connected, at this time, with the deeper vessels.

The further development is chiefly due to the multiplication of the lung units and the increase in volume of the interbronchial tissue. As new bronchi are formed, new groups of lymphatics bud off from the plexus that accompanied the parent bronchus and follow the new-formed structure towards the periphery. These lymphatics leave the bronchus and pass to the venous group when they reach the region where the air-sacs are developing.

As the lung-tissue differentiates further and further, the larger veins become more closely associated with the bronchi and only the terminal vessels are peripheral with reference to the lobule. This brings about the relations that are found in the adult, where the principal veins and bronchi are closely associated, while the terminal ones have the same relative positions that have been described for the developing structures.

The arteries in early stages lie very close to the bronchi and are associated with the plexuses that follow that structure. As these blood-vessels increase in size the bronchial plexus differentiates into two parts, following the arteries and the bronchi. This is accomplished by the growth of vessels around the arteries, and, as the artery increases in size, the two plexuses become entirely distinct, but are still connected by numerous anastomotic vessels.

In the meantime, the vessels of the pleura, which at from 5 to 6 cm. we have seen beginning to form the true pleural plexus, continue to proliferate, and thus form a fine-meshed plexus in the pleura between the blocking-off of the lobules.

The completion of the primary plexus is shown in figure 3, plate 1. This is the surface of the lung in a pig embryo of 6 cm. with the pleural vessels injected. Each of these uninjected areas represents a primary lobule, and the surrounding lymphatics mark out the connective-tissue plexuses. Figure 1, plate 5, illustrates one of the primary lobules, and the close-meshed plexus is the true pleural supply. It is still seen to be connected with the deep vessels of the septum.

Here and there one finds vessels passing from the terminal bronchi to the surface, in the lobule proper, to join with the fine-meshed plexus of the pleura. These pass around the air-cells, but are never found on their walls, and, uniting with the terminal vessels of the end veins, pass to join those in the pleura. These are the vessels described by Flint (1906) as seeming to dip down into the lobules from the pleura; these, he said, he could follow only a little way into the lobule. This is easily understood from the information gained from injections, for the vessels around the bronchi can not be seen in uninjected specimens, and consequently those which remain patent in sections seem to end abruptly in the midst of a lobule, whereas they in reality connect with those following the bronchi and terminal veins. The lymphatics that follow the terminal bronchi leave them just before the atria are reached and cross over to join the lymphatics which follow the veins. The lymphatics which accompany the veins pass to the pleura just where the veins bend to reach the center of the lobule.

Flint first observed the submucous plexus of the bronchi and trachea in embryos 23 cm. long. It was surprising that injections did not reveal this plexus very much earlier. I have not been able to demonstrate any lymphatics in the submucosa before the embryo reached a length of 19 cm. This plexus develops, as do all the secondary plexuses, by the outgrowth of vessels from the primary one and their coalescence to form the new group. This process has been carefully studied by Heuer in the formation of the mucosal plexus in the intestine. The submucosal plexus is complete just before birth and consists of numerous fine vessels that lie just beneath the bronchial epithelium. From this plexus numerous vessels pass down between the cartilaginous rings and join the lymphatic trunks which follow the bronchi, as has been described. In those bronchi having no cartilaginous rings there is only the one group of lymph-vessels to be found, and these have already been described.

The lymphatics of the adult lung were first described by Olaf Rudbeck in 1651–1654 (quoted from Miller, 1900). Since that time numerous workers have studied these vessels. In 1900 W. S. Miller reviewed the literature very thoroughly, and it will, therefore, be unnecessary to repeat that here. Miller studied the lymphatics in the lungs of adult cats and dogs by injecting them from one of the pleural vessels. He divided the lymphatics into four groups, as follows:

A. The lymphatics of the bronchi.
B. The lymphatics of the veins.
C. The lymphatics of the arteries.
D. The lymphatics of the pleura.

The lymphatics of the bronchi.—Miller describes two sets of lymph-vessels associated with those bronchi which have cartilaginous rings and only one with those which have no rings. In the former the two sets are connected by vessels that pass between the rings and join the trunks situated on the outer side of these structures. These trunks drain the lymphatics that accompany the smaller bronchi and empty into the nodes which are situated at the hilum of the lung. While there are several lymphatics accompanying the larger bronchi, only three are to be found with those nearer the air-sacs. These end by leaving the terminal bronchus just before it ends in the atria; one of them passes to the artery, while the other two join the lymphatics of the vein.

The lymphatics of the veins.—There is a single group of vessels that extends from the terminal vein to the hilar nodes. Along the larger veins there are several vessels, but the terminal ones are accompanied by only one or two lymphatics. Anastomotic vessels pass from the bronchial lymphatics to join those of the vein at each branching of the bronchial tree. The lymphatics that accompany those veins which go to the pleura join the pleural lymphatics.

The lymphatics of the arteries.—The lymphatics which accompany the arteries are very similar to those of the veins, with the exception that none of them pass to the pleura.

The lymphatics of the pleura.—There is only one plexus in the pleura, and this drains through several large trunks to the nodes at the hilum. There are anastomoses with the lymphatics of the veins, as has been said, but the drainage probably does not pass through these. Miller put his canula into a large pleural vessel and injected towards the hilum. After some time the deep lymphatics, as well as those of the pleura, were filled. He thought that the injection mass backed up into the deep vessels from the nodes at the hilum, since both the sets of vessels drain into the same nodes.

Miller does not confirm the findings of Sappey (1874) and of Councilman (1900) with regard to the interlobular lymphatics. Sappy thought that it was wrong to divide the lung lymphatics into superficial and deep groups on account of the rich anastomosis of these vessels. He thought that the lobules were surrounded by lymphatics which formed networks between the adjacent lobules in much the same manner as the blood capillaries do around the air-sacs. Councilman divided the deep lymphatics of the lung into two sets, the bronchial and the interlobular; the latter he interpreted as very important in infections.

While Miller does not agree with these observers in regard to the interlobular lymphatics, he does describe anastomoses between the lymph-vessels of the venous radicles and those of the pleura, and he emphasizes the peripheral location of the veins. It might well be that the vessels which Sappey and Councilman found in the interlobular septa were the lymphatics of the veins, since they did not have very accurate methods for the differentiation of these structures. It becomes more difficult to reconcile Miller's findings with those of Flint and the results of this study. Both Flint and I have found distinct groups of vessels in the interlobular connective tissue in embryo pigs. These groups of vessels are directed in their growth and location by the position of the veins, but are not limited in their distri-

bution to the venous trunks. The fact that so careful an observer as Miller does not find these lymphatics in the septa suggests the possibility that the assumption of mature activities in some way brings about the atrophy of all of the interlobular lymphatics except those that accompany the veins. Again, this plexus may be peculiar to the pig. It seems necessary that this question must remain unsettled until studied by some method other than simple injection.

The question of the drainage of the lung lymphatics is of exceptional interest and importance; and while we must depend, for the final settlement, upon physiological methods, there is much evidence available from morphological observations. In the larger vessels on the bronchi, the veins, and the arteries there are valves which point towards the hilum. This is assumed to be very good evidence that the flow is in that direction. No valves have been described in the lymph-vessels which accompany the smaller bronchi, veins, and arteries. Hence it can not be stated whether the lymph flow, in the lymph-vessels of the veins, is towards the pleura of the hilum; and, in like manner, the flow in the bronchial vessels might be either towards the hilum or towards the veins and arteries. With regard to the vessels on the pleura, all of the lymphatics above a certain regional level of the lower lobe drain either towards the mid-line and then course up in the pulmonary ligament to end in the nodes at the hilum, or pass by direct paths to these nodes. Those below this level drain to the nodes lying in the mesentery of the lesser curvature of the stomach. Some of these drain as do those above—towards the median and pass down in the ligamentum pulmonale—while others pass directly down from the posterior pole. This group of vessels which pass to the preaortic nodes drains about one-third of the lower lobe of the lung. This varies considerably; in some specimens as much as half of the lung has been found to drain in this direction.

This peculiar drainage of the lower lobes seems especially important from the bearing that it may have on the pathology of the lungs. It has long been known that the diaphragmatic vessels drain to these nodes, but there is no connection between these vessels and those of the lung proper. The lymphatics that pass through the pulmonary ligament apparently drain only the pleura; but, as has been shown, the deep lymphatics anastomose with those of the pleura, and therefore it seems possible for substances to pass from the lung-tissue to the preaortic nodes. What bearing this may have upon the pathology of the lungs or of the abdomen remains to be settled.

SUMMARY.

The lymphatics of the lungs are derived from three sources—the right and the left thoracic ducts and the retroperitoneal sac.

In embryos 2.6 to 3 cm. long, vessels bud off from the thoracic duct and grow across to the trachea, forming there a plexus that gradually extends over the ventral surface of the trachea, and especially down over the bifurcation. From this plexus vessels pass into both lungs and into the pleura.

The right thoracic duct divides, in embryos about 2.5 cm. long, into two vessels. One passes to the heart, while the other breaks up to form a plexus on the right lateral wall of the trachea. Some vessels from this plexus pass down into the hilum

of the right lung, while others anastomose with the plexus from the left side, which extends up over the trachea. The development of the lymphatics within the lung depends upon the division of the vessels into two groups—those associated with the veins and connective-tissue septa, and those associated with the arteries and the bronchi.

The former grow very rapidly, and following each of the branches of the pulmonary vein, pass to the pleura. There are at first only two or three lymphatics with each vein. In the early stages the terminal veins lie about midway between the adjacent bronchi, and in this plane a sheet of lymphatics develops from the vessels surrounding the veins and passes to the pleura, where they mark out the boundaries of the distribution of each bronchus. These vessels anastomose with those that grow direct to the pleura from the plexus on the trachea.

The bronchial vessels develop more slowly and at first are to be found only around the larger bronchi. As these structures increase in size and number, the lymphatics surrounding the main bronchi send vessels to the smaller ones and these form a plexus around each of the bronchi, so that the bronchial tree is surrounded by a continual series of branching tubes made up of lymphatic vessels. From every point of division of the bronchi, lymphatic vessels pass to the lymphatics of the veins; those around the terminal bronchus leave it near its ending in the atria, and pass to join the lymphatics of the veins or septa, or, more rarely, those of the pleura.

Lymphatics also arise from the retroperitoneal sac and grow up posterior to the diaphragm to enter the lower pole of the lower lobe of the lung. These vessels form a plexus on the median surface of the lower lobe, and send branches both to the pleura of the other surfaces and into the lung along the veins. Plexuses develop here as with those that come from above and the two groups soon anastomose.

The further development consists in the multiplication of the plexuses on the bronchi and blood-vessels, following their continued differentiation. As the lung increases in size, the larger veins become approximated to the bronchi and only the terminal ones are separated from them; these lie in the periphery of the lobule. Connective tissue is formed along the sheets of lymphatic vessels, and these become the septa of the lung, containing a definite set of vessels which develop from the early vessels following the veins. The lymphatics accompanying the veins remain connected with those of the bronchi and septa.

The common plexus surrounding the artery and bronchus is separated into two individual plexuses, incident to the increase in size of the artery; however, these continue to have anastomosing branches.

The vessels of the pleura mark out the early connective-tissue septa, but later there develops a fine-meshed plexus between these larger vessels, which is not connected with the vessels of the lung-tissue. The valves begin to form at about 6 cm. and, in general, point away from the pleura. None, however, have been found in the smaller vessels which accompany the terminal bronchi.

In the adult there are lymphatic vessels accompanying the bronchi, the arteries and the veins; these anastomose freely. There are also vessels in the connective-tissue septa which drain chiefly into those around the veins, and, to some extent,

into those of the bronchi and arteries, near the point where the vein and the bronchus separate to take their relative positions with relation to the lobule. There are numerous anastomoses between the deep vessels and those of the pleura, but probably most of the flow is towards the hilum. All the deep vessels, together with the greater number of the pleural vessels, drain into the nodes at the hilum; but the vessels of the lower half of the pleura of the lower lobe drain through several vessels to the preaortic nodes. These vessels pass through the ligament of the lower lobe and behind the diaphragm.

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Diagram of transverse section of left lung of an embryo pig 3 cm. long, in which the blood-vessels were injected through the umbilical artery with india ink. The lymphatics appear as dilated spaces (blue). The section is 20 μ thick and is stained with hematoxylin and eosin, aurantia, and orange G. ×55. *Ao*, aorta; *T*, trachea.

FIG. 2. Diagram of section through lobule of lung of an embryo pig 7 cm. long, in which the lymphatics were injected with india ink through the left tracheal plexus. The veins were slightly injected by the rupture of a lymphatic vessel into a vein near the hilum. The section is 100 μ thick and is unstained. ×47.5. *A*, artery; *B*, bronchus, *V*, vein; *Pl*, pleura.

FIG. 3. Surface of lung of an embryo pig 6 cm. long, in which the lymphatics were injected with india ink through the left tracheal plexus. The section was taken from the ventro-lateral surface of the left lower lobe and is about 200 μ thick and is unstained. ×29.4. *P L*, primary lobule.

FIG. 4. Longitudinal section of lung of an embryo pig 6 cm. long, in which the lymphatics were injected with india ink through the left tracheal plexus. The veins contain some blood pigment. The section is 400 μ thick and is unstained. ×33. *V*, vein; *B*, bronchus.

FIG. 5. Diagram of left tracheal plexus in an embryo pig 6 cm. long, in which the lymphatics were injected through the thoracic duct. Cleared by Spalteholz method. Note that part of the vessel marked with an asterisk (*) has been removed in dissecting the body-wall away. This vessel is the one described as the first to the lung. ×15. *, first vessel to lung; *T*, trachea; *Th D*, thoracic duct; *L T P.*, left tracheal plexus; *Ao*, aorta.

PLATE 2.

FIG. 1. Dissection of an embryo pig 4 cm. long, in which the lymphatics were injected with prussian blue through the retroperitoneal sac. The heart, aortic arch, left lung, and the body-wall have been removed. Cleared by the Spalteholz method. ×19. *Th D*, thoracic duct; *R Th D* right thoracic duct; *R T P.* right tracheal plexus; *L T P*, left tracheal plexus; *C L*, cardiac lymphatics; *Ao*, aorta; *B*, bronchus; *Oe*, esophagus.

FIG. 2. Section of a small area of the lung of an embryo pig 7 cm. long, in which the lymphatics were injected with prussian blue through the retroperitoneal sac. Drawing to show the relation of the peri-bronchial lymphatics to the wall of the bronchus. Section is 20 μ thick and is stained with hematoxylin and eosin, aurantia, and orange G. ×93. *B*, bronchus.

FIG. 3. Dissection of an embryo pig 4 cm. long, in which the lymphatics were injected with prussian blue through the retroperitoneal sac. The left lung, the arch of the aorta, the pulmonary artery, and the body-wall have been removed. Cleared by the Spalteholz method. The left tracheal plexus is shown as a solid blue mass because the meshes are so close that they could not be analyzed in the drawing. ×15. *Th D*, thoracic duct; *R Th D*, right thoracic duct; *Ao*, aorta; *L T P*, left tracheal plexus; *B*, bronchus.

FIG. 4. Longitudinal section of upper lobe of right lung of an embryo pig 6 cm. long, in which the lymphatics were injected with prussian blue through the retroperitoneal sac, and the veins were injected with india ink through the pulmonary vein. The section is 400 μ thick and is unstained. Cleared by the Spalteholz method. ×39.

PLATE 3.

FIG. 1. Small block of an embryo pig 15 cm. long, in which the lymphatics were injected with prussian blue by puncture of an interlobular septum. The arteries were injected with india ink through the pulmonary artery. Cleared by the Spalteholz method and mounted in balsam. The specimen was mounted at a convenient angle to best show the interlobular septum; unfortunately, it was jarred out of position while being drawn and hence the group of lymphatics in the septum is shown bent to one side. ×10. *Pl*, pleura; *A*, artery; *I L S*, interlobular septum.

FIG. 2. Diagram of a dissection of an embryo pig 3 cm. long, in which both the right and left jugular sacs were injected and, from them, the right and the left thoracic ducts respectively. India ink was used. The body-wall, heart, and left lung have been removed. Cleared by Spalteholz method. ×30. *Th D*, thoracic duct; *R Th D*, right thoracic duct; *V C S*, vena cava superior; *Ao*, aorta; *P A*, pulmonary artery; *C L*, cardiac branch of the right thoracic duct.

FIG. 3. Diagram of a section of the right lung of an embryo pig 6 cm. long. This is the same specimen from which figure 4, plate 2, was made; the part of the section shown in that figure is indicated by an X. ×20.

PLATE 4.

FIG. 1. Longitudinal section of the left lung of an embryo pig 5 cm. long, in which the lymphatics were injected with prussian blue through the retroperitoneal sac. The veins have some blood pigment in them. The section is 400 μ thick and is unstained. $\times 22$. *V*, vein.

FIG. 2. Dissection of an embryo pig 4 cm. long, in which the lymphatics were injected with prussian blue from the retroperitoneal sac. The right lung, esophagus, and body-wall have been removed. The stomach was pulled to the left side of the embryo in order to expose the retroperitoneal sac. Cleared by the Spalteholz method. $\times 20$. *Ao*, aorta; *L L*, left lung; *Th D*, thoracic duct; *D*, diaphragm; *R P S*, retroperitoneal sac; *, first vessel to the lung.

PLATE 5.

FIG. 1. Surface of lung of an embryo pig 23 cm. long, in which the lymphatics were injected with prussian blue, by puncture of an interlobular septum. Cleared by Spalteholz method. The interlobular septum is indicated by a very large lymphatic trunk. $\times 28$. *I L S*, interlobular septum.

FIG. 2. Longitudinal section of the upper portion of the lower lobe of the left lung of an embryo pig 5 cm. long, in which the lymphatics were injected with prussian blue through the retroperitoneal sac, and the veins have retained a little blood pigment. The section is 100 μ thick and is unstained. $\times 57$. *V*, vein; *A*, artery; *Pl*, pleura; *B*, bronchus.

FIG. 3. Lower portion of left lung of an embryo pig 5 cm. long, in which the lymphatics were injected with india ink through the retroperitoneal sac. Cleared by the Spalteholz method and mounted in balsam. $\times 28$.

Fig. 1 (C. L. 1 cm)

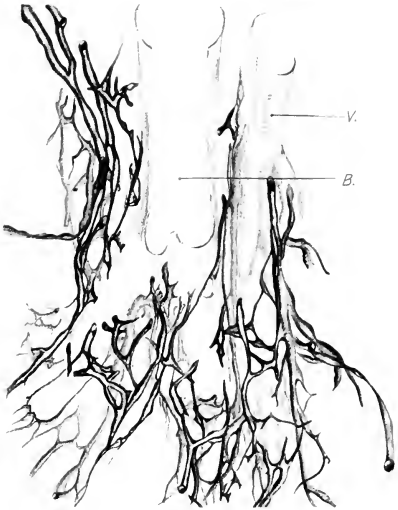


Fig. 4 (C. R. 6 cm)

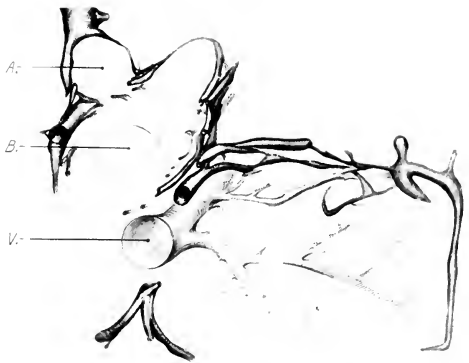
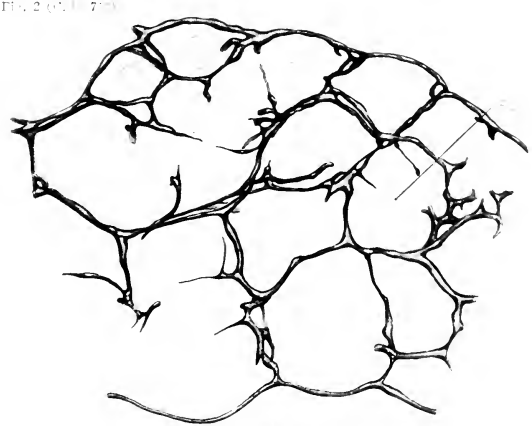


Fig. 2 (C. L. 7 cm)



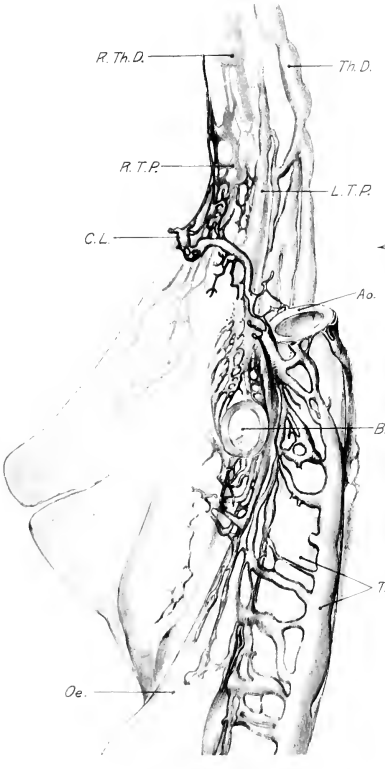


Fig. 1 (C. R. 4cm)

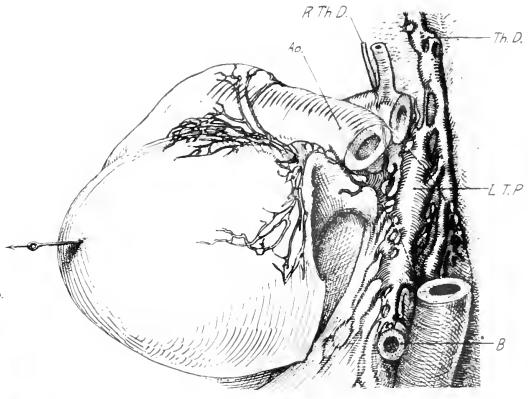


Fig. 3 (C. R. 4cm)

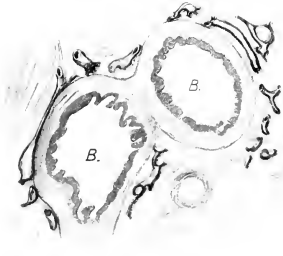


Fig. 2 (C. R. 7cm)

Fig. 4 (C. R. 4cm)

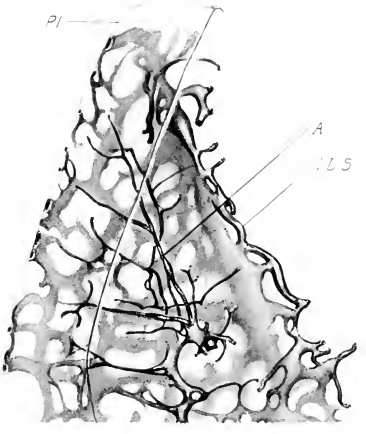


Fig. 1 (C. R. 15.m)

Fig. 2 (C. R. [illegible])

X

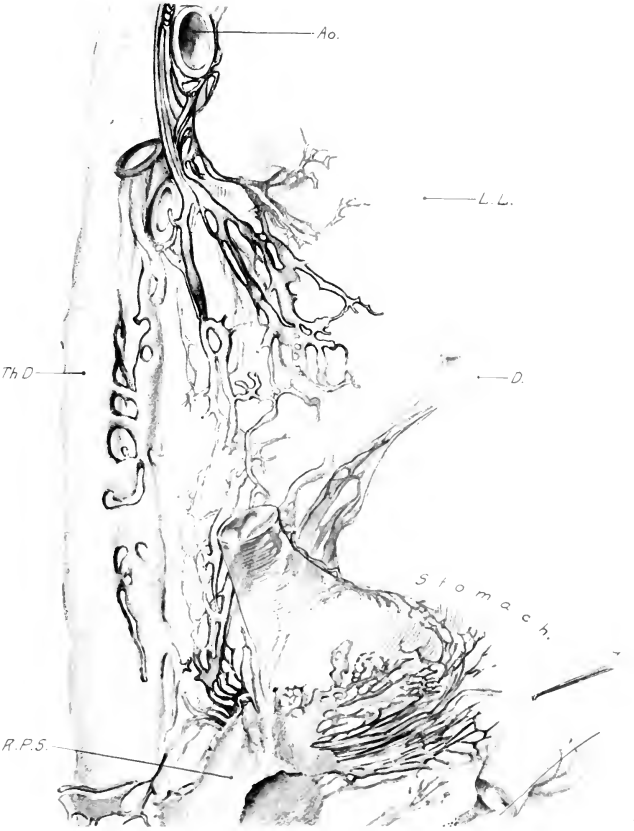


Fig. 2 (C. R. 4cm)

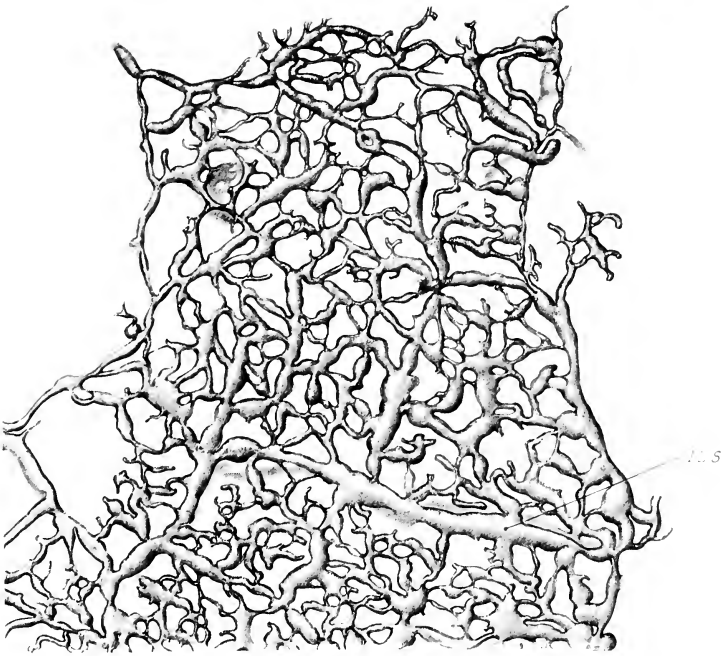
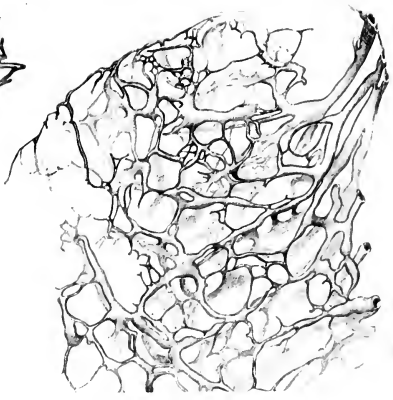


Fig. 1 (C. R. 23m)



CONTRIBUTIONS TO EMBRYOLOGY, No. 13.

BINUCLEATE CELLS IN TISSUE CULTURES.

By Charles C. Macklin.

Four plates, containing seventy figures.

CONTENTS.

BINUCLEATE CELLS IN TISSUE CULTURES.

By Charles C. Macklin.

INTRODUCTION.

In examining a living tissue culture, or a preparation from the same, one frequently finds a cell which contains two or more nuclei, of about equal size, slightly separated or in contact. In a communication of Lewis and Lewis (1912 *c*, fig. 12) a binucleate cell from a tissue culture is shown; more recently these authors (1915, p. 391) have referred to the occurrence of such cells in tissue cultures under the heading "Amitosis and giant cells." That they may be quite numerous in an area of new growth is seen by referring to figure 1, where one quadrinucleate and six binucleate cells appear in a small field.

The question of their origin and fate in cultures of embryonic tissue, involving as it does the idea of direct nuclear division, gathers interest from the fact that such cells are found in embryonic tissue developing *in vivo*, and from the further fact that they probably represent the first stage in the formation of certain giant cells. The problem of inquiring into their history by prolonged observation of the living cell was suggested by M. R. and W. H. Lewis.

METHOD.

Cultures were grown in ordinary hanging-drop preparations, the technique of W. H. and M. R. Lewis (1911, 1912*a*, 1912*b*, 1915) being employed. The tissue was obtained from embryo chicks of from three to ten days' incubation. Heart tissue was most frequently used, and gave very satisfactory results.

Locke solution as a culture medium was used. A stock saline solution was first made up as follows: NaCl, 18 grams, 0.9 per cent; KCl, 0.84 gram, 0.042 per cent; $CaCl_2$, 0.5 gram, 0.025 per cent; $NaHCO_3$, 0.4 gram, 0.02 per cent; H_2O, 2,000 c.c. Freshly distilled water and absolutely clean bottles are indispensable. The solution will keep apparently good for months.

Culture media was made up, from time to time, as required, from this stock solution, in 100 c.c. lots, by dissolving from 0.25 to 1 gram of dextrose in 100 c.c. of saline, thus making a solution of 0.25 to 1 per cent of dextrose. The media was then placed in clean plugged test-tubes, 10 c.c. in each, and sterilized in the Arnold sterilizer for 30 minutes, after which it was stored for use as required. It should be made up fresh every two weeks.

The best results were obtained by diluting this media, when it was being used, by the addition of 20 to 25 per cent of freshly distilled, sterile water, since some evaporation went on during the planting, and further concentration of the media often occurred in the preparation from evaporation of the hanging drop and conden-

sation of the vapor about the walls of the moist chamber on the depressed slide. By using a slide with a deep depression, containing a little distilled water, this evaporation was lessened.

Cultures planted in a hanging drop of this media, upon a sterile, clean cover-slip, inverted over a depressed slide, which was sealed with vaseline, grew very well at a temperature of 39° to 40°. However, it was found that if a small quantity of extract of chick embryo (Carrel, 1913) were added to the media better growths were obtained, *i. e.*, cells migrated out from the original piece earlier, growth was more rapid and vigorous, mitoses were more frequent, and a larger percentage of growths was obtained. Hence this addition to the media was generally made. Ordinary bouillon had a similar activating effect.

The embryonic extract was prepared as follows: After the embryo had been removed from the egg, under sterile conditions, and with as little contamination with yolk as possible, it was placed in a Petri dish containing 10 c.c. of sterile Locke's media and washed. The tissue to be planted having been dissected out and removed to another dish of media, the remainder of the embryo was cut up and placed in a small, sterile test tube with a little media, and carefully ground up with a glass rod. This mixture was next centrifugalized, and the supernatant fluid added to the culture, generally in the proportion of equal parts of this fluid and Locke. Too high a proportion of embryonic extract was undesirable on account of its richness in food material, in that it produced a cell overloaded with fat globules, which interfered with observation.

The advantages of glycosaline over plasma have been noted by Lewis and Lewis (1912*a*, p. 10). It is more transparent and practically all of the growth is upon the lower surface of the cover-slip—not scattered throughout the hanging drop, as in the case of the plasma clot. The cells, unimpeded by the fibrin network, migrate freely along the cover-slip, upon which they spread themselves flat and thin, thus facilitating observation. The quantity of fat being much less than in plasma-grown cells, the cytoplasmic constituents, such as the centrosphere and mitochondria, are much more easily observed and studied. There are, too, the additional very considerable advantages that the media is more convenient to handle, fixed preparations are more easily made and are not marred by stained fibrin and coagulated albumen, and experimental operations, such as staining with vital dyes, are more satisfactorily carried out.

Immediately after planting, the cultures were transferred to a warm box, kept at a constant temperature of 39 to 40 degrees by means of an electric thermoregulator. With the microscope inside this box it was not necessary to remove the cultures from their warm environment for purposes of observation, and it was owing to this manifest advantage that the earlier method of observation of the living cultures upon a warm stage outside the incubator was discontinued. In addition, the warm-stage method of heating, from one side only, was found to be inferior to that of the warm box, in which the culture was completely surrounded by an environment of uniform temperature.

Illumination was furnished by daylight, Tungsten globe, or Welsbach burner. A ray filter, consisting of a glass vessel filled with a solution of copper sulphate or

copper acetate, placed between the source of illumination and the condenser, when artificial light was used, was found to be an advantage (Kite 1913*b*, p. 149).

In studying the grosser changes, such as the variation in shape of the nucleus, the 4 mm. Leitz apochromatic objective was used; for the finer details the Leitz $_1/_{12}$ oil-immersion objective was found to be satisfactory. Oculars were Leitz Compensating Nos. 4 and 6.

For observation of the living cells the cultures of the second day were generally the most favorable; the growth was then usually abundant and the cells in a healthy condition, with a fair proportion of mitoses.

It is quite evident that continuous observation of the living cell, provided it can be carried out successfully, is the ideal method of studying the sequence of changes occurring therein. Indeed, for the study of amitosis it has been regarded as indispensable, as witness the statement of Richards (1911, p. 125): "For amitosis there is but one absolutely certain criterion, the observation of living material and subsequent study of material fixed under observation;" he adds, "this is, of course, impossible in most cases."

The method has already been used in the study of living multinucleate cells of tissue cultures, Lambert (1912*a*) having attempted to settle the question of the origin of giant cells growing from explanted tissue by its aid, and the character of the results attained through its use was sufficiently encouraging to warrant its application to the problem in hand, though not altogether satisfactory in view of the obvious difficulties. It was hoped, too, that these difficulties would be minimized by the use of glycosaline media, which produced films of tissue sufficiently thin for study in the living condition.

It was first planned to ascertain the full history of the binucleate cell by selecting a cell with a single nucleus and observing it continuously on the stage in the warm box till either the nucleus divided and formed a double-nucleated cell or the cytoplasm became merged with that of another mononucleate cell to form a single cell containing two separate nuclei. Observations upon this binucleate cell were then to be continued until the ultimate fate of the double nucleus was disclosed. Drawings were to be made from time to time with the camera lucida.

This ideal was found to be impossible of realization, on account of the technical difficulties. Cultures under continuous observation, exposed, as they were, to strong light, often showed evidences of degeneration; even daylight seemed to cause this and the use of ray filters did not altogether eliminate it. Degeneration was noticed at times even when the plan was followed of making short observations and immediately turning the light off, the culture remaining continuously on the stage.

Living cells show a marked tendency to migrate; hence the cell under observation had to be closely watched to prevent its escape from the field of vision. Other cells often wandered over the cell under inspection, and so interfered with the work. Added to these difficulties is the length of time involved in the process, which necessitates many hours—even days—of continuous observation. Then, too, the minuter cell changes are very difficult to follow, even for short periods, the only

optical picture presented being very delicate shades of difference in refractivity. The obscure and peculiar optical properties of living matter, as Kite (1913, p. 148) points out, give rise to an important source of error.

The ideal procedure having to be abandoned, the alternative practice was adopted of following shorter periods of change and piecing the records of these together. A start was made with the formation of the double nucleus, and here another difficulty was encountered; it was manifestly impossible to tell which of the thousands of nuclei in the culture was about to divide, and by selecting nuclei at random, months might be spent without getting one which ultimately divided. It was thus necessary to select a nucleus which gave some indication of being on the way to division, *i. e.*, by elongation, or equatorial constriction; such a cell was observed continuously until it divided or became degenerate. The subsequent history was studied by selecting a double-nucleated cell and observing it continuously.

In the observations the shape of the nucleus was particularly noted, and with this was considered the behavior of the centrosphere, mitochondria, fat globules, nucleoli, shape of the cell generally, and whether or not the cell itself ultimately divided following nuclear division. Cells on or near the outer border of the new growth were found most favorable, since they were larger and more free from surrounding cells. They appeared to be usually quite healthy during the first 48 hours at least.

As has here been noted, the morphology of the cell is difficult to make out in the living and unstained condition, and it was thought that inspection would be much easier and more accurate if the details could be rendered visible by the use of stains which would not impair the vitality of the cell.

Churchman and Russell (1914) and Russell (1914) have recorded satisfactory results with gentian violet in staining embryonic and adult tissues of the frog growing *in vitro*. They stated that endothelium from adult frog pericardium in frog's plasma to which gentian violet had been added grew definitely when the strength of gentian violet was 1 in 2,000, and actively in a dilution of 1 in 20,000. Furthermore, their records show that they were able to follow cell division in their stained preparations. Clear karyokinetic figures were not seen in growing adult frog tissue, but in embryonic frog tissue these figures were found in the dividing stained nuclei. They believe that the nucleus is stained intravitally and that growth continues in the presence of the dye. Toxic action was shown when this stain was used upon paramecium, even in dilutions of 1 in 1,000,000. They believe that "the use of stains in the plasma in which tissue is grown will probably facilitate the study of nuclear growth."

My results with gentian violet in chick tissue growing in Locke do not bear out those of Churchman and Russell, for the staining could not be considered as in any sense *intra vitam*, under the conditions existing in my experiments.

A solution of Grübler's gentian violet was made up in a strength of 1 in 100,000 with slightly diluted saline. Without removing the culture from the warm box, the cover-slip was lifted off and a small drop of the stain (warmed to the same tem-

perature as the culture and of about the size of the hanging drop) was added to the latter, the excess fluid being withdrawn. The dilution of the stain was thus approximately 1 in 200,000. The culture was immediately examined under the microscope. The dye rapidly diffused through the cytoplasm into the nucleus, the nucleoplasm taking on a finely granular appearance; this latter was, apparently, the result of the coagulative action of the dye. The nucleoli were distinctly marked out, and stained much more darkly than the nucleoplasm or cytoplasm. The nuclear membrane, too, was sharply outlined as a dark violet ring. This staining was very valuable in delineating indistinct nuclear boundaries, since these, in the living unstained culture, are often obscure. Irregularities in outline, such as indentations, were rendered very plain, and the method was of assistance in studying the relationship to one another of double nuclei.

The cytoplasm, after this treatment, consisted of coarse violet granules in a very faintly stained matrix, showing at times a slightly fibrous structure. Cell borders were well marked, especially the pseudopodia, which, however, lost their power of movement upon being stained. Intercellular bridges could be studied. Mitochondria were not specifically stained, and degenerated in a short time. In a culture so stained evidence of life, such as pseudopodial and mitochondrial movement, cell migration, and mitosis, ceased almost at once, and in a few minutes vacuoles formed in the cells and the entire culture became degenerate.

Though gentian violet staining is of great assistance in obtaining a conception of the morphology of the cells rapidly, under the conditions of the experiments its toxic action precludes the possibility of the stained cell undergoing vital changes. Owing to its coagulative action the appearance of the living protoplasm is not accurately reproduced in the stained preparation. In spite, however, of these disadvantages, the use of gentian violet enables one to inspect portions of the cells which, in the living condition, are almost, or quite, invisible, and also to examine more accurately and easily some of the visible parts.

M. R. and W. H. Lewis (1914 and 1915, p. 376) used janus green as a vital stain in tissue cultures growing in Locke solution, and found that, although the mitochondria were specifically stained, the dye was toxic in as low dilutions as 1 in 200,000, and caused speedy death of the cells, as well as distortion of the mitochondria.

Janus green (Hoechst), di-ethyl saffranin azo di-methylanilin, in Locke's solution, in a strength of 1 in 40,000, was applied to living cultures in the same manner as the gentian violet, and was found to stain the mitochondria specifically in about 5 minutes, but no movements of these bodies could then be noted, and the threads broke up into a row of granules. The cells soon died, as evidenced by their vacuolated and degenerated appearance.

While janus green staining provided a rapid and convenient method of observing mitochondria, its toxic action rendered it valueless as a means of studying vital changes; moreover, the stained mitochondria soon lost their normal optical characters, thus prohibiting extended observation.

Some of these living cultures were fixed and stained. Osmic-acid vapor was used for fixation, and Heidenhain's iron hematoxylin was found to give the best staining.

The culture, growing in the hanging drop, was removed from the vaselined slide and exposed to the fumes from a 2 per cent aqueous solution of the tetroxid of osmium. This may be done by placing the cover-slip, drop down, over the mouth of a bottle containing the fixative (the vaseline adhering to the cover-slip and preventing the escape of the vapor) or, as suggested by M. R. Lewis, by floating the cover-slip, drop up, upon the osmic solution. Fixation is complete in 5 or 6 minutes, and the preparation is then dark brown or black. It is now rinsed off with distilled water, and passed rapidly through ethyl alcohol solutions of 35, 50, and 70 per cent. To the latter a few drops of hydrogen peroxid are added, which bleaches the preparation. It is then passed back rapidly through the same alcohols, rinsed in distilled water, and washed in running tap-water for 5 minutes. Too long immersion in alcohol will cause the mitochondria to become dissolved out.

The cover-slip, culture downward, is now floated upon a solution of 4 per cent iron alum and allowed to remain for 12 to 24 hours; next it is washed in running tap-water for 5 minutes and then immersed in 0.5 per cent aqueous hematoxylin for 24 to 48 hours, after which it is washed in running tap-water for 1 minute, differentiated in 2 per cent iron alum and again washed in tap-water for 10 minutes, dehydrated through the alcohol series, cleared in xylol, and mounted in balsam. The hematoxylin solution is prepared as follows: Hematoxylin (10 per cent in absolute alcohol), 0.5 c.c.; distilled water, 10.0 c.c.

These fixed preparations were used to make clear the morphology of the living cells, especially such details as nuclear membranes, nucleoli, mitochondria, and centrosomes. An attempt was made to pick out the successive stages in the process of direct division of the nucleus for comparison with the observations upon living material, and thus to build up a series exhibiting the various changes. The phases of mitosis were also studied, and drawings were made of interesting cells. For statistical purposes cell counts were made of some of these preparations by placing a glass disk, upon which squares had been ruled, in the ocular, and using the mechanical stage.

THE BINUCLEATE CELL.

INCIDENCE.

The frequency of occurrence of binucleate cells varies within wide limits in cultures from different tissues. They were found to be most numerous in membranes growing from the heart, and were not uncommon in cells of the connective-tissue type from this and other tissues, but in the endodermal membranes from stomach and intestine they were exceedingly rare. They may be even altogether absent from the new growth. Lewis (1915, p. 156) notes that in growths from the leg of chicks no amitotic forms were noted.

To get an idea of the relative number of these cells as compared with the total number of cells in the new growth, careful counts were made of 20 fixed cultures from chick heart. Imperfect cells and those situated so close to the original piece as to be indefinitely outlined were omitted. In these 20 preparations there was a total of 41,725 cells, of which 375 were binucleate, or an average of 1 binucleate to

each 111 cells; thus the binucleate cells made up 0.9 per cent of the total cells appearing in the new growth.

Even in different preparations from the same tissue binucleate forms were found with varying frequency. Among the 20 cultures of heart mentioned above, one preparation showed 1 double nucleus to each 28 cells, while in another the ratio was 1 to 1,180.

Age of tissue, too, in these 20 heart preparations, had a bearing upon the incidence of binucleate forms, new growths from the younger hearts showing a somewhat greater proportion of double nuclei than those from older cardiac tissue. In hearts from chicks of 5 days' incubation there was, on the average, one binucleate to each 105 cells; in 7-day hearts the ratio was 1 to 123, and in 8-day hearts it was 1 to 233.

Finally, duration of growth seemed to be related to the relative frequency of occurrence of these cells. In the same 20 preparations it was found that cultures of the first 24 hours showed one double nucleus to each 183 cells; in cultures of the second 24 hours the ratio was 1 to 86 cells. This seems to point to a considerable amount of nuclear splitting in the second 24 hours, of which some at least probably occurred within the new growth. Cultures of older duration were, in the slides counted, not sufficiently numerous and typical to base accurate conclusions upon.

MORPHOLOGY.

The average binucleate cell (figs. 1*a*, 7, and 9) is somewhat larger than the average mononucleate, the area occupied by the nucleus being approximately twice as great. Each nuclear part is, in size, shape, and general appearance, very similar to the nucleus of the mononucleate cell. The nuclear parts are often pressed close together (figs. 1*a*, 60) and their adjacent surfaces are consequently flattened, the intranuclear pressure in each being evidently equal. When thus related, the appearance of the double nucleus in the living preparation (and indeed in some of the fixed preparations) simulates a single nucleus which looks as though it were separated by an equatorial membrane. Such an appearance has been interpreted as a nuclear plate, or intranuclear membrane, and so described by Child (1904, 1907*b*, and 1911, p. 283), and others; but for reasons which will appear later on, I believe that such appearances in tissue cultures are due to the apposition of nuclear surfaces, as above described.

In an elongated nucleus which has become bent upon itself the folded free edge of nuclear membrane, projecting into the karyoplasm, may simulate a partition which seems to be growing across the nucleus from one side to the other. A nuclear configuration of this character is presented in Child's (1911) figure 16, page 293, and in other of his figures. It is not to be wondered at that the approximated areas of nuclear wall at the folded edge are somewhat attenuated and appear thin, as Child (1911) has observed (p. 283). Such reduplications of nuclear membrane are not to be looked upon as intranuclear membranes which cleave the nucleus by growing across its equator. I have seen no evidence of a type of amitosis of this kind.

Sometimes an equatorial membrane is simulated by an elongated nucleolus lying across the nucleus. Again, as Richards (1911, p. 124) suggests: "A strand of

linin stretched across a nucleus with chromatin granules upon it often gives the appearance of a membrane dividing the nucleus amitotically (endogenous division?)." He also states that he has found no evidence of the "endogenous" division of Child (1907*a*, p. 95); nor have I seen anything of this kind in tissue cultures. Optical appearances similar to Child's (1911) figure 6 have been seen in living cells and interpreted as indentations and infoldings of the nuclear membrane. All these conditions can be made clear by the use of a dye like gentian violet upon the living culture, or by proper fixation and staining. In no case has a *bona fide* intranuclear membranous partition been found in any kind of preparation.

I may also state here that my observations upon fixed and stained cells in tissue cultures have not disclosed cases where one nuclear half was more darkly stained than the other, such as those mentioned by Child (1904, p. 549; 1906, p. 595; 1907*c*, p. 171; and other places) and which he believes to indicate "a certain degree of physiological independence before separation of the parts." In the living condition, too, the nuclear portions present no evident difference in cytoplasm. The contents of the nuclear parts are in every way similar to those of the single nuclei. The nucleoplasm appears homogeneous during life and when fixed with osmic-acid vapor is finely granular. This method of fixation preserves most accurately the details of the living cell (Lewis and Lewis, 1915).

There is usually at least one nucleolus or karyosome in each nuclear portion, and more often two (figs. 7 and 9) or even more. The nucleoli of the connective-tissue type of cell are irregular in shape, often elongated, and vary greatly in size (fig. 8). In the living cell they are highly refractive. They continuously undergo changes in shape, size, and number during the life of the cell (figs. 24 to 35, and plate IV), as can be seen by watching the living nucleus. It is then apparent that their outline is "ragged," as Lewis and Lewis (1915) describe it. The bodies even appear to break up from time to time, and afterward to recombine (figs. 24 to 35). At times the nucleolus comes to lie very close to the nuclear membrane (fig. 29) and it may even appear to be attached to it. These bodies take the gentian violet dye very well and stain darkly with hematoxylin. If overdifferentiated with iron alum the nucleolus appears as an agglomeration of small granules of about equal size (fig. 10); it is probably to be regarded as a gel of varying density, the densest portions being represented by these darkly staining granules.

During mitosis the nucleolus disappears with the formation of the spireme, and the daughter nucleoli reappear in the reorganizing daughter nuclei. The nuclear portions may be separated by an interval (fig. 9), or simply touching one another (fig. 7), or may be pressed so close together that their adjacent surfaces are flattened, similar to the condition in the early cleavages of *Moniezia*, as mentioned by Harman (1913, p. 221). They tend to remain close to one another, and do not migrate far apart, as nuclei in a syncytium. When separated, the nuclear portions show mitochondria between them (fig. 9) and usually the centrosphere is situated either in the interval between the nuclear portions, or opposite this interval, as in figures 7 and 59.

In the living condition the centrosphere or "central body" of Lewis and Lewis (1915) appears as an area of slightly greater refractivity situated at one side of the nucleus in mononucleate cells; this side is frequently concave, with the centro-

sphere situated in the concavity (fig. 24*c*). This concave side then appears indistinctly marked out in the living culture, the close proximity of the centrosphere and mitochondria obscuring the nuclear outline. Its relation to the parts of the double nucleus has been noted.

I have not observed the centrosome (centriole) in the living cell, but when stained with iron hematoxylin this body appears usually as two minute dark granules, lying close together (fig. 7). The centrosphere takes a slightly darker stain than the area surrounding it, and thus appears to be a somewhat more concentrated area of the protoplasm. From this area mitochondria radiate, as seen in figure 8. In the living condition the centrosphere shows an indefinite, irregular, apparently serrated edge, the toothlike processes of which undergo a curious constant, slow, almost imperceptible indrawing and outpushing. The mitochondria seem to be intimately connected with this body, as observed by Lewis and Lewis (1915, p. 349), but they differ from it in their reaction to janus green and to certain methods of staining in the fixed condition, such as iron hematoxylin.

Mitochondria in tissue cultures have been described at length by Lewis and Lewis (1914, 1915). Their curious movement, mentioned by these authors, is plainly evident. The special relation of these bodies to the binucleate cell is their position between the nuclear portions, as in figures 8 and 9, unless, as in figure 7, the parts of the nucleus are too close together to permit of this. The relationship of the mitochondria and adjacent centrosphere to the portions of the double nucleus is similar to that of the Netzapparat of Deineka (1912, figs. 2 and 12) under similar conditions.

Fat, though not so abundant as in plasma-grown cultures, nevertheless occurs as fine globules which tend to crowd together at the nuclear poles (fig. 32) and often become arranged in rows between the mitochondria.

The other details of the binucleate cell are very similar to those found in the mononucleate.

Occasionally cells are found which contain three or more distinct nuclei (fig. 1*b*) and the evidence seems to indicate that the binucleate cell is the first stage in the formation of the giant cell; this stage, however, is seldom passed, for giant cells are comparatively rare. Such multinucleate cells are quite different from the foreign-body giant cells of Lambert (1912 *a* and *b*), which have been shown by him to arise by fusion of previously separate wandering cells.

Binucleate cells, and the intermediate stages leading up to them, have long been known in embryonic tissue. Child (1907*c*) shows several such from chick embryos in his figure 12. Maximow (1908) describes and figures double nucleated cells, similar to those found in tissue cultures, in mesenchyme of embryo rabbits of $11\frac{1}{2}$ to $13\frac{1}{2}$ days, and he has found amitosis also in the guinea pig in the same region and stage. Patterson (1908) shows illustrations of cells of the same type in developing pigeon's eggs, and such cells have been described by many others. Thus it is certain that, since the paired nucleus occurring in the tissue-culture cell is similar to that found in the cells of embryonic tissue, it can not be considered as an abnormality due to its artificial mode of life.

Harrison (1913, p. 67) has shown that the behavior of cells growing in culture media is comparable to that of cells growing in the embryonic body, and it is reasonable to assume that the behavior of these binucleate cells in tissue cultures approximates the behavior of similar cells in the developing embryo. Hence the vital phenomena manifested by such binucleate cells in tissue cultures afford reliable evidence as to the changes which take place in similar cells living under normal conditions in the corresponding embryo.

ORIGIN.

If we inquire as to the origin of these binucleate cells of the new growth we are confronted with four possibilities, viz:

(*a*) Migration as a binucleate cell from the explanted tissue.
(*b*) Fusion of the cytoplasm of two previously separate cells without fusion of the nuclei.
(*c*) Division of the nucleus by mitosis without division of the cytoplasm.
(*d*) Division of the nucleus by amitosis without division of the cytoplasm.

This list does not include the theoretical origin of nuclei *de novo* from the cytoplasm, or their development from extruded chromidial substance (Young, 1913). These hypotheses do not appear to have been substantiated, and no evidence in favor of either appears in tissue-culture preparations.

First, considering (*a*), we find that twin nuclei occur in the area of new growth immediately surrounding the original tissue, and such forms are well known in embryonic tissue. Thus it is probable that many of the binucleate cells in the new growth have migrated as such from the explanted tissue. The great increase in proportion of double-nucleated cells in the second 24 hours, however, as has been noted, suggests that not all of these cells are of migratory origin, but that some have probably arisen in the new growth itself. This view is borne out by observations upon the living cell, as will be shown, where a single nucleus has been seen to become divided directly into two parts, and also by the finding of nuclei in the act of direct division in the fixed preparations.

The binucleate cells which have migrated as such from the original piece have probably originated therein in the same manner as those arising in the new growth. Giant cells can hardly be considered to have migrated as such from the original piece, for in the zone immediately surrounding the latter they are not found.

Regarding (*b*), it may be said that no appearances which could be interpreted as transitional forms have been found in fixed and stained preparations or in cultures vitally stained, neither has the process been observed in the living culture. I therefore regard it as an improbable hypothesis. This could hardly be considered as an explanation of the formation of giant cells, for that would postulate the fusion of a multitude of previously separate cells, of which there is no evidence in the material examined.

It may be noted that Lambert (1912*b*), who brought about the formation of giant cells by fusion of mononuclear cells in cultures from chick spleen, failed to get such cells in cultures from chick heart. Furthermore, Lambert (1912*a*) recognized three other types of giant cells in tissue cultures, besides this.

Considering next (*c*), we find that this also is improbable. It is easy to observe the process of mitosis *in vitro*, and to follow the various changes. Many such cases have been observed, and in none has there been seen a failure of the cytoplasm to divide following separation of the chromosomes. This process of cytoplasmic constriction is well shown in figures 68, 69, and 70, and in the living culture it is very evident and easy to watch. In no case has it been observed, in following these cells dividing by karyokinesis, that a binucleate cell was formed; always the end result was two distinct daughter cells, often widely separated, connected by a thin strand of protoplasm (fig. 1, *t*). If crowding of the cells occurs, separation of the daughter cells may be interfered with to some extent, but it is doubtful if this interference ever is so serious as to prevent cytoplasmic fission altogether and thus result in the formation of a single cell containing two nuclei. At least no evidence has been found from observation of the cells of tissue cultures that this is ever the case.

Upon this point my observations agree with those of Child (1911, p. 283). He says: "In *Moniezia* nuclei which arise by mitosis are separated by an appreciable distance when they form." Again (p. 292), in describing a "double" nucleus, represented in his figure 11, he says: "The two parts of the nucleus . . . are in immediate contact and flattened against each other. It is difficult to understand how they could attain such a position as the result of mitotic cleavage, like that of the earlier stages."

It must be said that my observations upon living cells have principally been made with cells of the connective-tissue type. In the case of membranes, however, there is always a well-marked dividing line between the cells, which is made evident by staining with iron hematoxylin or the use of silver; also this potential isolation of the cells is made apparent by the fact, when cells do separate, that the cleavage is along this line of partition, as is shown from the study of fixed preparations (Lewis and Lewis, 1912*c*, figs. 14, 13, and 12). No such partition is ever found between the nuclear parts of binucleate cells.

In fixed preparations of connective-tissue cells there is no indication of any failure of the cytoplasm to divide in the later stages of mitosis; that is to say, we find no telophases where separation of the cytoplasm is not evident (fig. 17).

Again, these double nuclei almost always have only a single centrosphere (fig. 7), whereas nuclei arising by karyokinesis have each a centrosphere. This finding as to the centrosphere agrees entirely with that of Deineka (1912) for the Netzapparat in the dividing epithelial cells of Descemet's membrane and connective-tissue cells of the cornea. This author is of the opinion that the Netzapparat surrounds the centrosome, and its changes appear to follow the variations of the latter body. In binucleate cells of these tissues, in which the nucleus divides by amitosis, the Netzapparat remains single, whereas if the nuclear division takes place by mitosis each of the daughter nuclei obtains a separate Netzapparat. By reference to this disposition of the Netzapparat, Deineka is even able to tell the manner of origin of such double nucleus, whether by amitosis or mitosis in which cleavage of the cytoplasm has been delayed. I have never observed this cell organ in living tissue-cultures.

The fact that the centrosphere in the binucleate cell is single seems to indicate that the twin nucleus is single so far as its reproductive capacity is concerned. This inference is borne out by observations, later to be referred to.

Considering finally (*d*), it seems probable that these twin nuclei arise through direct equal binary fission of the nucleus without division of the cytoplasm. The evidence upon which this assumption rests is, first, the inadequacy of other explanatory hypotheses; and, second, the observation in living cells of a process which is apparently direct nuclear division, and the occurrence in fixed preparations of tissue cultures of what must be regarded as transitional forms between single and double nuclei.

It is true, as Harman (1913) remarks (p. 219), that "the fact that two nuclei lie in contact is no evidence that they have arisen by amitotic division," and in the material which she studied, viz, early cleavage stages of *Tænia teniaformis* and *Moniezia*, she undoubtedly presents convincing evidence that nuclei which have arisen by mitosis may lie quite close to one another within the same cell. This, however, is a case of delayed cleavage, for she states (p. 215):

"In cleavage, nuclear division takes place very much in advance of cytoplasmic division. In the early divisions it is the exception and not the rule to find even a constriction in the cytoplasm. This gives rise to a syncytial condition. This syncytium persists until very late cleavage."

This is quite a different condition from that obtaining in the cells of tissue cultures. Then, too, many of her nuclei contain spiremes. Her contention in no way counts against the view that the double nuclei of tissue cultures are of amitotic origin.

Observations on direct nuclear fission will now be recorded, first to be described being the process as it was seen to occur in the living cell. As has been pointed out, it is impossible to tell from inspection of the living culture which of the thousands of mononucleate cells will divide directly, and so to follow the process of nuclear amitosis in the living cell it is necessary to select a cell which shows some indication of beginning direct division, *i. e.*, by elongation and constriction. Figures 24 and 25 appear to be typical of the early stages of direct division of the nucleus.

Many attempts to trace the changes in such a cell were made, with, however, only partial success, for in almost every case the nucleus lost its constriction and became rounded again, or the cell degenerated. However, one case was found where what appeared to be direct division of the nucleus occurred during observation. The various phases are shown in the series of figures 24 to 35, which were drawn at 15-minute intervals from a single cell growing in a culture from a 5-day chick heart in Locke solution with extract from chick embryo. The culture was of 57 hours' duration. A cell was first selected which contained an elongated nucleus with a marked notch in one side. In this notch the centrosphere was situated, and consequently this side was somewhat indistinctly outlined (24). Instead of dividing, the cell straightened out, almost losing the indentation (25). It contained two nucleoli, one situated in the uppermost pole, and the other, which was paired, about the equator. The nucleus next became rounded (26 and 27) and, after one hour's observation, its outline was almost circular (28). In the latter figure there appeared to be only a single paired nucleolus.

The nucleus now became elongated and a refractive mass appeared in the lowermost pole—apparently another nucleolus; at the same time the central nucleolus became a single mass, and was somewhat longer than before (29). Next, a shallow notch formed in one side, and the nucleus became shorter and thicker, its nucleoli undergoing minor changes (30 and 31). At the end of two hours the nucleus again elongated and a deep notch appeared, indistinctly marked out on one side (32). This seemed to become shallower in 33, but the presence of the centrosphere prevented this portion of the nuclear membrane from being well defined.

The next change was the formation of another notch on the opposite side, both notches forming what seemed like a zone of constriction about the nucleus. A refractive mass stretched across the equator of the nucleus between these notches (34). This is apparently a strand of mitochondria rather than a nucleolus, for, in the next drawing (35), $2\frac{3}{4}$ hours after the observation began, this strand is situated between two apparently separate nuclear portions, the nucleus having divided directly. In no fixed and stained cell has a nucleolus been seen to occupy this position; on the other hand, mitochondria have frequently been seen between these nuclear parts, as in figure 8. There was here no evidence of the formation of either a spireme or an amphiaster, and thus Wilson's (1900) criterion for amitosis was fulfilled. It may also be noted that the centrosphere did not divide and the nuclear membrane remained intact.

The final division apparently took place very rapidly, since the actual separation was completed in the 15-minute interval between 34 and 35. This rapidity of the end process of nuclear cleavage accounts for the infrequency of such terminal constricting forms as figures 6 and 8, and makes the relatively small number of these later transitional forms adequate to account for the number of binucleate cells which originate therefrom. The cell was allowed to remain on the microscope stage all night, but unfortunately wandered away and was lost, so the subsequent changes could not be followed. The drawings were made from direct observation, but not with the aid of the camera lucida. Mitochondria and centrospheres are partially diagrammatic. This process, though traced with difficulty, and though somewhat obscure, seems to follow the classic descriptions of amitotic division of the nucleus, viz, elongation with equatorial constriction, forming a somewhat dumb-bell shaped figure, and final separation of the two nuclear portions.

A similar elongated nucleus in a connective-tissue cell was followed for $6\frac{1}{4}$ hours, and did not divide, but finally degenerated; in the meantime it underwent various changes in shape and was rounded when last observed. The changes in nucleoli were similar to those in figures 24 to 35.

Thus it appears that a nucleus in a condition of elongation and constriction may remain undivided for a long time and may even return to the rounded form without dividing at all. In cases, however, where the constriction has passed a critical point, as apparently was the case in the nucleus represented in figure 34, the process of division proceeds rapidly.

The study of fixed preparations, too, throws some light on nuclear amitosis, for in these one frequently finds nuclei evidently undergoing direct division. Such

forms are to be regarded as transitional stages between the mononucleate and binucleate cell. Figure 2 shows a nucleus which has undergone elongation and equatorial constriction, so that there is an indentation on either side. The nucleolus appears to be dividing also; this condition of the nucleolus is, however, not constant. Figure 3 shows a cell in which constriction is somewhat farther advanced; here the nucleoli have apparently divided, two being seen in each nuclear portion. In these cells the method of preparation does not show cytoplasmic details.

Figures 6 and 8 show nuclei in which direct division is almost complete, the nuclear parts being held together only by the finest filament. Similar nuclear figures were found by Maximow (1908) in embryonic rabbit tissue, as shown in his figure 1, and the upper two nuclei in his figure 10. In figure 8 the nucleus has divided unequally, and the larger portion contains two nucleoli, while the smaller has but one. In figure 6 only one nuclear portion contains a nucleolus, and this is single. In both cells the unchanged centrosphere is situated in its characteristic position between the two nuclear portions, while the mitochondria radiate out from this body, and a strand of mitochondria passes over the bridge connecting the nuclear parts.

There is nothing in the appearance of these nuclei to suggest the late telophase of an intranuclear mitosis, such as those shown by Cary (1909) and referred to by Richards (1911, p. 158). The clearness characteristic of the cells of tissue cultures prevents confusion of nuclear amitosis with the late telophase of mitosis, such as has been shown by Richards (1909) to be possible in the cells of *Tænia*.

In figure 9 nuclear separation has been completed, the two portions being quite free from one another. These are of about equal size and appearance, and each contains two nucleoli. Mitochondria and centrosomes occupy their typical positions; the former are short rods, this being a cell from heart membrane.

In figure 7 the separate nuclear parts have come together and their surfaces are just touching. Mitochondria have been forced out, but the centrosphere is characteristically opposite the area of contact of the nuclear portions. Figure 4 shows a somewhat similar binucleate cell, from a Zenker and Mallory preparation.

It would appear that the nucleus may sometimes divide by a gradually deepening cleavage from one side, which finally cuts it into two pieces. This may be regarded as an asymmetrical type of constriction. Figure 5 may be taken as representative of the beginning of this process and figure 6 the end. The centrosphere is found typically in the notch, as has many times been recorded in amitotically dividing nuclei, as by Maximow (1908). In the rare exceptions to this rule the centrosphere may have been originally situated in the notch and subsequently have left it. No evidence of separation of the centriole-pair during nuclear amitosis has been found.

Richards (1911, p. 156) finds constricted and indented nuclei in his material only in cases of imperfect fixation. Whatever may be said as to the nuclear distortion brought about by many fixatives, this is not an explanation of such figures as 6 and 8 seen in tissue cultures, for here osmic-acid vapor was used as a fixative and this does not change the nuclear outline, as may be proved by observing a living nucleus and the same nucleus after fixation (Lewis and Lewis, 1915). Then, too, only a small proportion of nuclei appear thus, whereas if the appearances were to

be interpreted as due to the fixative they should be abundant. Again, the actual observation of such nuclei in living cells is proof absolute that they are not artifacts.

The only type of nuclear fission which I have observed in tissue cultures is that which occurs, apparently, by constriction.

An estimate of the frequency of occurrence of such transitional amitotic nuclear forms as those shown in figures 2, 3, 6, etc., was made by making careful counts, the aforementioned series of 20 heart cultures being used. Out of a total of 41,725 cells in this series, 50 cells were found to contain constricted nuclei of such a character as would warrant their being considered as amitotic. This is a proportion of one amitotic nucleus to 835 ordinary nuclei, or 0.1198 per cent. In the same series there were 375 binucleate cells, which are regarded as end products of nuclear amitosis. The proportion of transitional forms to end products is thus 50 : 365, 1 : 7.5 or 13.33 per cent. So high a percentage of transitional forms seems to indicate that the nuclei remain a long time in this condition, and the observations upon living cells bear this out. The final stages of direct nuclear fission, as shown in figures 6 and 8, are, as has been noted, rarely found.

In this connection it is of interest to compare the incidence of amitotic with that of mitotic nuclei. In the same series there were found to be 170 cells undoubtedly in mitosis. The ratio of mitotic cells to total cells is thus 170 : 41,725, or 1 : 245, or 0.4 per cent. There were probably many more mitoses than this, for some are undoubtedly rubbed off in preparation, since their rounded and thickened form exposes them to friction in washing, etc.; also 62 doubtful mitotic forms were not included.

It is an easy matter to calculate the relative proportion of amitotic to mitotic forms. As has been stated, the ratio of amitotic nuclei to total cells is 1 : 835, while that of mitotic nuclei to total cells is 1 : 245. It is evident that the mitotic forms are 3.4 times as numerous as the amitotic, even when we leave out the doubtful forms and the cells in mitosis which have been rubbed off. Again, when we consider that the amitotic process is a slow one, as has been shown, and that mitosis is relatively rapid ($1\frac{1}{4}$ to $2\frac{1}{2}$ hours according to Lewis and Lewis, 1915, p. 371), it will be realized that the amitotic method of nuclear division is unimportant, so far as nuclear multiplication is concerned, as compared with mitosis.

Thus, examination of living and fixed preparations makes reasonable the view that direct division of the nucleus occurs where this structure is elongated, and sometimes bent upon itself, by a karyoplasmic streaming, away from the nuclear equator, and a gradually deepening constriction which encircles the nucleus more or less symmetrically and cuts it into two parts, the constricted area becoming a narrow tube and finally a thread, which ultimately disappears.

The behavior of the nuclear membrane during amitosis in the cells of tissue cultures seems to be essentially the same as that of the same structure in the cells of the trematode described by Cary (1909) during intranuclear mitosis. There is, however, no intranuclear spindle in the cells which I have examined.

The final separation of the constricted nucleus takes only a short time, as has been noted, but a nucleus may remain for a long time apparently about to divide without actually doing so.

In the process of direct division of the nucleus various factors may play a part. First, we may refer the different changes in form of the nucleus to changes in form of the cell as a whole. It is of frequent occurrence that a cell, by reason of the tension exerted by attached cells, or of its own amœboid movement, becomes elongated. In consequence of this stretching of the cell the nucleus also becomes drawn out, it being simply a sac of fluid, and it is possible that it may become broken into two parts much in the same manner that an oil globule, floating upon water, becomes broken up if stretched. It may be assumed that there is a streaming of protoplasm away from the equator, with a constriction in this region, which becomes deeper and deeper until the nucleus is divided into two more or less equal portions, these now tending to assume a more globular shape. This view of the cause of nuclear amitosis is somewhat similar to that of Maximow (1908), who believes that amitosis in the mesenchyme cells of developing rabbits may be brought about by the stretching of such cells consequent upon rapid growth of the adjacent liver.

The process of direct division of the nucleus as described is strikingly like the division of the cytoplasm of ova which had been replaced in normal sea-water after having been treated with hypertonic sea-water (J. Loeb, 1906, p. 66, figs. 10, 11, 12, and 13). It appears that here the cell first becomes incut from one side; the protoplasm thereupon streams off in opposite directions, forming two globules connected by a narrow isthmus. This soon becomes reduced to a mere thread composed of the attenuated cell membrane, which finally disappears, so that there remain two sacs of protoplasm, quite without connection one with another. The physical changes involved in this process seem to be very much like those seen in direct division of the nucleus. Loeb's figures are very similar to those illustrating nuclear amitosis.

That the size of the nucleus is not a material factor in this process is seen by the variation in size of the twin nuclei, some of which are quite small. Although a twin nucleus is frequently found in a cell which is not elongated, it may be assumed that such a cell has subsequently changed its form, but that it was extended when the separation of the nucleus occurred. This hypothesis would not, however, explain the formation of giant cells, multinucleated muscle-cells, etc., and it does not provide an explanation for the evident activity of the centrosphere and mitochondria in direct division.

A second hypothesis to account for the separation of the nucleus directly postulates the active participation of the centrosphere, or mitochondria, or both, and here we may assume a purely mechanical and a purely physico-chemical activity. It has been noted that the centrosphere is found commonly in the invagination of the nucleus; moreover, its edge shows evidence of a curious type of movement—a slow, indefinite retraction and elongation of the marginal processes—which seems to be associated with movements of the mitochondria. It is possible that, through this mechanical influence of the centrosphere upon the adjacent nuclear membrane, the constriction of the latter is favored and the nucleus ultimately divided, and it is easy to conceive how the mitochondria may assist in this nuclear separation

through their own movements (as they have been described by Lewis and Lewis, 1915), since they are typically found between the nuclear parts when these are separated to any extent (fig. 9), and a strand of mitochondria may even be seen lying across the constricted isthmus of the nucleus, when this has not become completely divided (figs. 6 and 8). This position of the centrosphere and mitochondria undoubtedly seems to have some significance in separation of the nucleus, and is seen even where the nucleus is dividing irregularly, as in figures 48 to 58.

The relation of the Netzapparat of Deineka (1912, fig. 3) to the nucleus is similar to that of the centrosphere as just described, viz, it is found in the cleft separating the nuclear portions. This author, however, does not ascribe to it any function in nuclear separation. He believes that it surrounds the centrosome.

The position of the centrosphere and mitochondria may, of course, be without significance, so far as the actual division of the nucleus is concerned, and it is possible that the relationship of these cytoplasmic bodies to the amitotic nucleus is purely fortuitous, or, at most, occasioned through their adjustment to conditions of intracellular pressure. The occasional absence of the centrosphere from the cleft (once in each 50 cases as determined by counts) and the presence of a cleft opposite the one in which the centrosphere is found are points which count against this second hypothesis. Again, not all nuclei, in which the centrosphere appears in a concavity on one side, divide directly; indeed, this relationship of centrosphere and nucleus has frequently been noted and illustrated in cells developing, without nuclear amitosis, in their normal environment. It would seem, therefore, that this relationship, of itself, can not bring about nuclear amitosis.

In no case has there been noted a ring-shaped centrosphere, like that described by Meves (1891), which encircles the constricted zone in the dumb-bell-shaped, amitotically dividing nucleus.

The centrosphere and mitochondria may be assumed to act in another way in accomplishing direct division of the nucleus, viz, by bringing about a change in the surface tension of the area of the nucleus to which they are opposed, through the elaboration of a chemical substance, and it may be possible to explain direct division of the nucleus upon some such hypothesis as that used by Robertson (1909, 1911, and 1913) to account for division of the cell in mitosis, viz, that there is produced in the region of cleavage some chemical substance which lowers the surface tension, such as soap, and that there results, in consequence, a streaming of protoplasm away from the equator, leading to separation of the cell. Robertson postulates a cholin-fatty acid soap, the cholin being derived from the splitting-up of lecithin. Since it has been shown that mitochondria are lecithinoid bodies (Cowdry, 1914, p. 18) it is not beyond the range of possibility to assume that they may act in the formation of a cholin soap. Indeed, the relation of mitochondria to the production of cholin in nerve-cells has recently been discussed by Cowdry (1915). The position of the mitochondria, lying across the zone of nuclear constriction (fig. 8) is eminently favorable for the action of such a soap, should it be formed there.

A third theory to account for direct division of the nucleus is based upon the assumption that some intranuclear change inaugurates the process. As long ago

as 1855 and 1858. Remak set forth a theory to account for the division of the cell, which may be stated in the words of Wilson (1900, p. 63) as follows:

> "Cell-division proceeds from the center toward the periphery. It begins with the division of the nucleolus, is continued by simple constriction and division of the nucleus, and is completed by division of the cell-body and membrane."

A type of division which bears a close resemblance to this has recently been described by Howard and Schultz (1911) in the cells of a giant-celled sarcoma from the human œsophagus. To this type of division Schultz (1915) has proposed the name "promitosis," and these investigators believe it to be intermediate between amitosis and mitosis. This form of cell division seems to have an interesting parallel in certain protozoa, and they regard it as a reversion to a primitive biological condition in which the division sphere is permanently intranuclear—an idea analogous to that of Wieman (1910, p. 175) for a similar form of nuclear division.

The first step in the division of the nucleus here is taken to be a separation of the karyosome into two or more parts, of equal or unequal size, followed by a breaking-up of the nucleus into portions corresponding in number and size with the fragments of the karyosome, each nuclear part coming to contain a portion of the latter. This function of the karyosome in initiating division of the nucleus is analogous to that of the centrosome in mitosis.

This form of nuclear division is essentially the same as that described by Conklin (1903) in the follicular epithelium of the common cricket, and that it is by no means infrequent is gathered from the numerous references to it which this author has found in the literature. Conklin, however, has never seen actual cell division following nuclear amitosis, and from the fact that the cells in which direct division of the nucleus is found speedily degenerate after the egg is laid he believes that it is, in the material examined, "one of the last functions of these cells and that it is therefore an accompaniment of cellular senescence and decay." Conklin, however, believes that in most cases of amitosis the nucleolus does not divide.

The evidence from tissue-culture cells does not lend much support to a hypothesis ascribing to the fission of the nucleolus the initiation of nuclear division; true, we have in figure 2 a nucleus which shows lateral constrictions at the equator, and within it, lying with its long axis parallel to that of the nucleus, is an elongated karyosome, which also appears to be undergoing division in the same plane as the nucleus. This somewhat resembles the nuclei described by Howard and Schultz; the karyosomes of tissue-culture cells, however, are decidedly simpler in structure than those of the cells of the giant-cell sarcoma. Again, the fact that in the binucleate cell each nuclear portion is usually supplied with one or more karyosomes seems to point to this body having been divided before or during the division of the nucleus; but against this circumstance, weighing in favor of the view that the division of the karyosome acts to excite direct nuclear division, is the occurrence of such division where the karyosome has evidently not divided (fig. 6), since it is present in only one of the nuclear parts. Such nuclei are not uncommon in tissue cultures. The peculiar condition of the nucleolus in figure 2 may thus be purely accidental, since it is not at all constant.

The division of the nucleolus thus seems to have nothing to do with the separation of the nucleus; indeed, after the nucleolus has divided, the nucleus may not divide at all. It may, however, have to do with the size of the nuclear portions; where these latter are equal they each contain one or two nucleoli, of about equal size, whereas where they are unequal one portion—usually the smaller—may not contain a nucleolus.

That direct division of the nucleus may take place without preliminary fission of the karyosome in tissues developing normally is evident from the statement of Wilson (1900, p. 115): "In many cases, however, no preliminary fission of the nucleolus occurs; and Remak's scheme must, therefore, be regarded as one of the rarest forms of cell division." It is interesting to note that Schultz finds evidences of such a simple form of direct division in the nuclei of cells of the same tumor in which he finds "promitosis."

Summing up, then, the process of direct nuclear fission, it is probable that various factors are involved. Elongation of the nucleus is undoubtedly sometimes followed by its cleavage, and, since it is always present in nuclear amitosis, it may be regarded probably as an essential in this. The activity of centrosphere and mitochondria must also be considered as a factor in equal, as well as unequal, nuclear fission, and this activity is apparently made effective by nuclear elongation.

Fission of the nucleolus, while possibly concerned with the relative size of the nuclear parts, is not necessarily associated with the initiation or carrying out of nuclear cleavage.

Inasmuch as binucleate cells, and constricted nuclei which must be regarded as their precursors, are found in apparently normal embryonic tissue, they can hardly be considered as abnormal or as evidence of a reversion to a more primitive type of cell division; furthermore, their healthy condition is manifest from their capacity to divide by mitosis, as will be shown hereafter. Thus it is reasonable to suppose that the factors operative in nuclear division in tissue cultures are those which function in embryonic cells *in vivo*.

Since these binucleate cells seem to represent the first step on the road to certain giant cells it may be concluded that the latter are the result of a repetition of the same processes which bring about the formation of the former. This view is in accord with that of Lewis and Lewis (1915), p. 391, who state: "These giant cells appear to be formed by an amitotic division of the nucleus without a coincident division of the cytoplasm."

FATE.

The nucleus having divided directly, what becomes of it? Obviously the most certain method of settling this question is to select a living binucleate cell and watch it constantly as it passes through its various changes. This course has been followed with several cells, and the evidence at hand does not show that the cell as a whole divides otherwise than by the regular process of mitosis; in the early stages of this process there is a combination of the two nuclear portions to form a single mitotic figure.

Plate IV is a series of camera-lucida drawings representing successive stages in the history of one of these twin nuclei, in a living connective-tissue cell, grown from

a 7-day chick heart in glycosaline with autogenous embryonic extract, the culture being 19 hours old when the observation commenced.

At $11^h\ 55^m$ a. m., when the observation began, the nucleus (fig. 60) was seen to be composed of two portions, approximately equal, separated by what appeared to be a single membrane, but what really represents, as has been shown, the apposed areas of nuclear membrane of the two portions. This double partition was seen, by focusing at different levels, to be a plane surface. The first three drawings show roughly the appearance of such a double nucleus during life. The parts are of about the same size and each at first contains a single nucleolus. These latter undergo obvious changes in size, shape, and number. There is a single centrosphere (c). Fat globules are numerous, and the mitochondria are thread-like and plainly visible, and show their characteristic movement.

The nucleus remained in much the same condition, undergoing minor changes in outline, for about 2 hours, when, at $1^h\ 50^m$ p. m. (63) the division between the nuclear parts was seen to become less clearly defined at one side and, gradually, refractive material from the nucleus accumulated in this equatorial plane until, at $5^h\ 05^m$ p. m. (65), there was a distinct refractive mass in this region, which was evidently chromatin. Soon the entire cell began to contract, to become rounded, and to draw in its processes; the nuclear outline became indistinct, the position of the nucleus being represented by a clear space surrounded by a ring of fat globules and mitochondria (66). By focusing up and down it is seen that the cell is much thicker than before—in fact, it is almost spherical, the mitochondria and particles of fat forming a hollow globe which incloses the nuclear space. The portions of the twin nucleus have quite evidently fused and (from our knowledge of mitosis) it is plain that the cell is now in the prophase. A spireme, however, could not be made out. The refractive material which had been seen between the nuclear portions has become indistinct. This stage was seen at 6 p. m.

If we could see the cell represented in 65 in the fixed and stained condition we would doubtless find something like figure 22; here the spireme is forming in a binucleate cell and the nucleoli are becoming smaller and are breaking up. It is evidently composed of two such nuclei as are seen in figure 14, an early prophase in a mononucleate cell. The accumulated chromatin in the plane of contact of the two nuclear portions is clearly evident; this is obviously not the equatorial plate of mitosis. The nuclear membrane has almost disappeared, but the chromatic material is somewhat more concentrated about the periphery.

Figure 23 evidently represents a somewhat later stage of spireme formation in a double nucleus. Here the skein is well marked and the nuclear membrane has completely disappeared. These figures bear a striking resemblance to figure 6 of Rubaschkin (1905), in which he shows a spireme in a double nucleus.

The stage represented in 66, if fixed and stained, would probably resemble figure 19, drawn from a mononucleate cell in the late prophase. From this point on the behavior of the combined double nucleus is identical with that of an ordinary single nucleus.

As the cell was watched it was seen that a line, refractive in character, formed across its equator; this line, represented in 67, was somewhat irregular in outline, its borders being serrated. It did not remain unchanged, but on the contrary

showed almost constant minor variations in contour; it seemed to be composed of a row of small refractive bodies (chromosomes) undergoing constant, slow, and very slight movements. From this characteristic formation, situated as it was in a diamond-shaped field, surrounded, as before, by a granular ring of refractive globules and mitochondria, the metaphase of mitosis was easily recognized. This stage was drawn at $6^h\ 50^m$ p. m. (67) and would appear like figure 15 if fixed and stained. The cell is somewhat smaller and more condensed than that seen in 66, and the appearance plainly indicates that the centrosome has divided and that each part is performing its usual function at a pole of the spindle. The actual division of the centrosome was not observed.

After a short time the plate was seen to split, and the two halves, retaining their parallel relationship to one another, moved to opposite poles of the cell, and there remained, thus marking the anaphase. Figure 16, from a fixed preparation of a mononucleate cell, represents this stage. Almost immediately thereafter the granules and fat globules midway from the poles of the cell were seen to move inward as though a constriction were occurring about the nuclear area at this zone; the result was a dumb-bell-shaped mass within the elongated cell, formed of the nuclear area and surrounding protoplasm. Almost at once the cell membrane itself was seen to be undergoing constriction at this point, as shown in 68, at $7^h\ 05^m$ p. m. At the same time the nuclear areas at either end of the cell commenced to become free from granules of fat and other refractive material and the cell outline became larger, showing that the cell was flattening out and that the daughter nuclei were becoming reconstituted in the telophase.

That the intracellular pressure is considerably increased during this process is shown by the bulging outward of certain portions of the cell membrane, as illustrated in 68, to form bubble-like protuberances. Frequently the granules and fat globules may be seen to rush out into these evaginations, indicating the formation of cell currents, where pressure has been suddenly released, through giving way and stretching of localized areas of the cell wall. These protuberances soon flatten out, lie close to the cover-slip and expand, becoming armed with hyaline borders possessed of amœboid movement (Harrison, 1913, p. 67). The end of the cell opposite the connection with the daughter cell thus appears fimbriated, as shown by Lewis and Lewis (12*c*, figs. 8 and 10). These refractive borders act as pseudopodia to anchor the cell to the cover-slip and to drag the daughter cells apart.

The reforming nuclei, now more widely separated, and showing wider and clearer areas in the cell protoplasm, are seen in 69 at $7^h\ 25^m$ p. m., and at this time the cell was very much constricted, with the nuclei more widely separated. The constricted zone is somewhat more highly refractive than the surrounding tissue and resembles a short thread. Here also the cell processes are seen to be feeling their way outward and to be pulling the two daughter cells apart. The stage corresponding to this in the fixed preparations is shown in figure 17; here the chromatin is a closely clumped, darkly staining mass, and the individual chromosomes are becoming resolved into smaller granules. These subsequently become scattered, and appear in the later definitive, more lightly staining, nucleus as in figure 18. A marked expansion of cytoplasm is here to be noted.

There have thus been formed two separate and distinct daughter cells, in each of which the nucleus is becoming gradually reconstituted. As the cell was watched the nuclear areas became clear and the membranes distinct; nucleoli also appeared, two in each nucleus. Separation of the cells continued, their hyaline borders becoming very active, stretching away into the outlying media and writhing in a sluggish, eel-like manner. Soon the fat globules took up their characteristic arrangement in the cytoplasm, mitochondria appeared, and, in 70, at 8 p. m., 8 hours after the observation commenced, we have to recognize two cells, apparently normal, each with its own centrosome.

The process of mitosis was identical with that followed many times in mononucleate cells, except for the variation in the introductory stage, occasioned by the formation of the spireme from two nuclear parts instead of one. The various stages of mitosis, as it is found in the mononucleate cell, are well shown in the series, figures 14, 15, 16, 17, and 18, selected from a fixed preparation.

I have been unable to ascertain whether such spindle formations arising from the fusion of two nuclear portions are possessed of a double number of chromosomes, but the apparent identity of the mitotic process, after nuclear fusion has taken place, with that occurring in mononucleate cells, does not suggest any material variation in the chromatin arrangement. I am in agreement with Maximow (1908), when he says regarding similar spiremes (p. 95): "Aus diesen Spiremen entstehen immer regelmässige normale Mitosen."

These cells were not followed farther. The history for the period of 8 hours, however, shows conclusively that spiremes from these double nuclei may combine to form a single equatorial plate and division may occur by the ordinary mitotic process. That such mitosis occurs in all cases it is impossible to state from this isolated observation, but the presence of double nuclei (with spiremes like those shown in figures 22 and 23) here and there in the fixed preparations no doubt points to the occurrence of such nuclear fusion as a part of the process of mitotic division in the binucleate cell.

Cases have not been found where one portion only of a bipartite nucleus was in a condition of mitosis; hence it seems reasonable to conclude that both parts are always involved in the process. This much is demanded by our conception of the potential unity of the double nucleus, so far as its reproductive capacity is concerned.

In the case of the cells from which figures 22 and 23 were drawn, it may be argued that these represent telophases in which the daughter nuclei failed to separate. Many mononucleate cells have been followed entirely through the mitotic process, and failure of the daughter nuclei to separate has never been noted. Again, in figure 23, drawn from an iron-hematoxylin preparation, there is only a single centriole-pair, not two, as would be the case in a telophase.

It might even be suggested that such daughter nuclei have recombined, as observed by Kite and Chambers (1912); here, however, artificial conditions were existent, since the cells were being forcibly separated in the Barber moist chamber by mechanical means. Moreover, entire absence of constriction of the cytoplasm, as would occur in the telophase, points to the condition we are considering as representing the prophase. More than this, the fact that the process has been followed

in the living cell, from resting twin nucleus through mitosis to two separate and distinct daughter cells, would seem to be proof absolute that these figures 22 and 23 (which represent a phase of this process) are prophases of combining double nuclei.

The mere contact of two spireme-bearing nuclei (such as appear in figure 23), is of itself no evidence that they will combine, but when we bring to bear upon the interpretation of such a figure the evidence derived from a series such as that shown in plate IV, in which a nuclear formation, like that of figure 23, represents a stage, it seems obvious that these nuclear parts are undergoing fusion to form a single plate of chromosomes. Harman (1913) shows several figures of such nuclei in early cleavages of *Tænia taniaformis* and *Moniezia* (her fig. *c*, plate 8), but here the separate nuclei have arisen by mitosis, according to her observations, and cleavage, which is delayed, will eventually separate the blastomeres. The nuclear membranes are here quite intact, and show no evidence of beginning dissolution.

It may be objected that the condition of spireme is no indication that mitosis is beginning. To this the reply may be made that in the cells of living tissue-cultures a nucleus showing a spireme of this kind, no companion cell in the same condition being present, always represented the prophase of mitosis.

Since mitosis occurs in binucleate cells *in vitro*, it might be assumed that it would also occur in such cells *in vivo*, and indeed this is the case, for Maximow (1908) has found figures in fixed preparations from the mesenchyme of embryo rabbits which strongly resemble those just described. In his figure 7 (p. 93) the spireme is forming in a dumb-bell-shaped nucleus, and in his figure 8 the nuclear fragments in which the spireme is found are quite separate. Maximow believes that his pictures represent the prophase of normal mitosis occurring in amitotic nuclei; this belief is supported by my observations upon the living cell shown in plate IV. In his figure 8 he finds the centriole-pair situated between the two coils of the spireme—a position corresponding to that characteristic for it in the amitotic nucleus, viz, in the cleft. In my figure 23, which is slightly later, the centrosome has shifted its position to the pole. He states that his results resemble the findings of Karpow (1904) for urodele amphibia; this latter author described a process of nuclear amitotic division, with subsequent formation of a spireme from the fragments (which may be two or more in number), with fusion to form one "mutterstern." It may therefore be concluded, from the finding of such double spiremes in embryonic tissue, that this process of mitosis in binucleate cells occurs in normal development. It is thus to be found in differentiating as well as non-differentiating cells.

I regret that I have seen no other living examples of combination of the parts of a double nucleus during mitosis, but the process is so rare that its observation thus is largely a matter of chance hitting upon a favorable cell. Mitosis occurs rather infrequently in the mononucleate cell, and when it is considered that the proportion of binucleate cells to total cells is very low (1 to 111) the remoteness of the possibility of finding a binucleate cell which will divide by mitosis may be realized. It is only in those cultures showing abundance of both binucleate cells and mitotic figures that there is any hope of finding such compound mitoses.

To ascertain the relative frequency with which mitosis occurred among the binucleate cells, as compared with the mononucleate, a study, by careful counting

and classification of cells, was made of the 20 preparations from chick heart mentioned before. In these estimates only the prophases were counted, since it is impossible to say of the cells in the later stages of mitosis whether they arose from a monopartite or bipartite nucleus. Degenerate cells were omitted, and also the area close to the original piece was not counted, since the cells here were usually too small and closely packed to be seen clearly. Nuclei with more than two parts of equal size were rare; such were grouped with the binucleate cells in this estimation.

It was found that there was a total of 41,106 mononucleate cells (excluding the later mitotic and amitotic forms); of these 47 were in the prophase of mitosis, or 0.114 per cent of the total. In the same series there was a total of 375 binucleate cells, 2 of which were in the prophase, or 0.53 per cent.

In spite of the rarity of occurrence of binucleate cells in prophase (there being only 2 in a total of 41,725 cells) it will be seen from this result that mitosis occurred even more frequently among the binucleate cells than among the mononucleate—in fact, 4.65 times as frequently. Thus, while it can not be stated definitely that mitosis with recombination of the nucleus always follows amitotic nuclear division, or, indeed, that it frequently does, it may nevertheless be affirmed with confidence, even allowing for the limited extent of the observation, that the incidence of mitosis in the binucleate cells is at least as high as that among the mononucleate.

If, in addition to this division by mitosis which these binucleate cells show, they be considered as also proliferating by direct division of the cytoplasm, it will be readily seen that their rate of proliferation would then be very much greater than that of the mononucleate cells. The improbability of this excessive multiplication strengthens the negative evidence to be put forward later that there is, in these binucleate cells, no division of the cytoplasm following direct division of the nucleus.

We have seen that a single mitotic figure can be formed from two nuclear portions, previously separate, but contained within the same cell. It has also been found that the spireme may form in a bent nucleus of a shape similar to those undergoing direct division. Figure 20 represents an early spireme in such a nucleus. There is apparently but a single centrosphere, situated in the cleft. Figure 21 shows a somewhat more advanced spireme. The nuclear membrane has disappeared and the chromosomes are more definite. One centrosphere is situated above, in the cleft, and there is an indistinct trace of a second in the clear area below.

We can thus construct a series, from cells taken from fixed specimens, illustrating prophases in single nuclei, in double nuclei, and in the intermediate forms connecting these. Figures 14 and 19 show spiremes in single nuclei. In the last are two well-marked centrospheres, indicating that a spindle is about to be formed. Figures 20 and 21 show the process in intermediate forms, and figures 22 and 23 show it in the double nucleus.

In figures 20 and 21 it is reasonable to suppose that the amitotic process has ceased, since the nuclear membrane has almost or quite disappeared, and for the same reason the process of karyokinesis, which is so obviously taking place in these cells and in those represented in figures 22 and 23, must in all of these cases be considered as starting up under circumstances where amitosis of the nucleus was under way, or was completed, rather than as having the process of amitosis superposed upon it.

Altogether the various forms of the nucleus in which spiremes are found in tissue-culture preparations resemble strikingly the findings of Karpow in the leucocytes of urodele amphibia. This similarity is brought out in the following paragraph from Maximow (1908, p. 95):

"Nun ist es aber nach Karpows Untersuchungen ziemlich sicher, dass hier die Kernamitose zwar zur Kernpolymorphie und sogar zu sicherer Kernteilung führt, dass sie aber doch keine richtige Zellvermehrung nach sich zieht. Wenn die Leukocyten mit amitotisch zerschnürtem Kern sich teilen, so geschieht dies eben auf dem Wege der Karyokinese, und aus einem zerschnürten Kern oder sogar aus mehreren einzelnen, ganz getrennten, durch Amitose erzeugten Kernen entsteht dann eine einzige, gewöhnlich regelmässige, mitotische Figur. Man findet Spireme in ring-, hantel-, rosenkranzförmigen Kernen, oft auch zwei oder mehrere einzelne Kerne in einer Zelle, alle gleichzeitig im Zustande des Spirems, woraus dann immer ein gewöhnlicher Mutterstern resultiert."

Maximow also shows a spireme in a dumb-bell-shaped nucleus found in his own material, and observes:

"Die tief eingeschnürten, oder auch schon ganz zerteilten Kerne können in Mitose treten und man bekommt dann hantelförmige Spireme (fig. 7) oder zwei kugelige Spireme nebeneinander in ein und derselben Zelle (fig. 8)."

Thus it would seem that the nucleus enters upon the process of mitosis whenever the stimulus initiating this process occurs, whether rounded, bent, undergoing constriction, or divided into two parts, and in all of these, after the single spireme has been formed, the process is apparently identical.

The question as to whether or not the cytoplasm of the cell divides following direct fission of the nucleus, to form two separate and distinct cells, has been much discussed by various authors, among them Maximow (1908), who found —besides the cases in which the amitotically divided nuclear portions formed a single combined mitotic figure and divided by karyokinesis—also instances where such portions simply became separated from one another and surrounded by protoplasm, to form ordinary mononucleate cells. In short, Maximow believes that, though amitosis of the nucleus may be followed by cell division arising through a process of mitosis involving the directly divided nuclear fragments, yet it can lead directly to cell proliferation without intervening mitosis. As such a method of actual cell multiplication, Maximow believes that amitosis functions in certain areas of the normal developing tissue of the rabbit, and he has found it also in the guinea pig. Furthermore, he expresses the view that cells arising by direct division can later divide by mitosis, but his reasons for the latter assumption are not given.

On the other hand there are those who oppose this view and believe that nuclear amitosis is never followed by cell amitosis. For instance, Karpow (1904), according to Maximow (1908, p. 89) came to the conclusion, based upon his observations upon the leucocytes of urodele amphibia: "dass in den Fällen, wo richtige Amitose wirklich vorliegt, man eigentlich doch nur Kernvermehrung annehmen kann, keine Zellvermehrung." This view is in agreement with the findings of Conklin (1903, p. 671) for follicular epithelial cells of the common cricket.

No reliable evidence that fission of the cytoplasm follows that of the nucleus has been found in the tissue cultures examined by me. It is quite true that so-called "paired" cells (*i. e.*, cells closely resembling one another in form, staining, etc.,

lying side by side, but separated by cleavage of the cytoplasm) may be picked out in the fixed preparations, and it might be urged that such were of amitotic origin. This contention can not be proved, however, and it is more probable, in view of the lack of positive evidence of amitotic division of the cytoplasm, that these cells are either of mitotic origin or have migrated together.

The problem was attacked by the method of continuous observation of binucleate cells (in which the double nucleus has been shown to arise by direct fission), the object being to see if the cytoplasm would divide, and in this way give rise to two separate mononucleate cells. Several such cells containing twin nuclei were followed, but in every case the cell finally degenerated without dividing, after an observation of shorter or longer duration. As an example, the following may be recorded: In a connective-tissue binucleate cell from an 8-day chick heart of 24 hours' growth, the portions of the nucleus were at first pressed closely together, but after 30 minutes they separated slightly, as in figure 9, and remained apart for 2 hours, when they again became pressed together. The cell was observed for $11\frac{1}{2}$ hours, and the process of separation and reapproximation of the nuclei occurred four times during this period. There was no trace of cytoplasmic division and the only changes noted were those mentioned — some shifting of position of the nuclei and a slight decrease in size of the nuclear parts; the latter is believed to be due to prolonged exposure to light. Continuous change in shape of the cell was followed by change in shape of the nucleus.

This observation shows conclusively that the binucleate cell may remain a very long time without direct division of the cytoplasm, and has been confirmed in the case of other binucleate cells. In living cultures the absence of evidence of direct division of the cytoplasm, combined with similar absence in the case of fixed preparations, leaves us with no ground for the assumption that such direct division ever occurs. Even granting that cytoplasmic division occurs at all, the process appears to be so long delayed that it can not be of much importance as a method of cell proliferation.

This view is in accord with that of Conklin (1903, p. 670), for follicular epithelial cells of the common cricket, but does not coincide with that of Child (1907, *c*, *d*, and *e*), who concluded from this examination of the cells of *Moniezia* and other animals that amitosis was a rapid method of division which occurred where the stimulus to divide was very great and the supply of nutrition was inadequate. Patterson (1908) and others hold similar views. From the evidence which tissue cultures afford, however, I am inclined to agree with Harman (1913, p. 219) that the assumption that amitosis is a more rapid method of cell proliferation than mitosis is hardly justified.

The observation just recorded also shows that the interpretation of "double" nuclei (such as those seen in my figures 4, 59, and 60 as separate nuclear sacs touching one another) is correct, for the sacs have been seen to move apart and afterward to return to their original contact with one another, and to repeat this process. As has been already mentioned, the apposed surfaces of such paired nuclei give rise to an appearance resembling an intranuclear plate; such a plate has, however, not been found by me in the cells of tissue cultures.

The twin nucleus is, then, to be regarded as potentially a single nucleus, in which the nuclear material is separated into two or more sacs. This nuclear material is not to be considered as in any way equally divided between the nuclear portions, which are by no means daughter nuclei. This view is strengthened by the fact that the centrosome, as has been observed, is single in binucleate cells. Before the cell containing such a single twin nucleus can divide, it seems to be essential, judging from the observations, that the nuclear material should recombine and a spireme be formed from the chromatin material in its entirety.

It may be asked whether nuclear fusion, in these binucleate cells, ever occurs without an accompanying mitosis. I have seen no evidence of such recombination, either in living or fixed preparations, and regard it as improbable, because (among other reasons) the parts increase in size following their division and the single nucleus, which would result from their reunion, would be unusually large.

Nothing was brought to light, in the material examined, which would in any way support the assumption that there are two distinct types of cell division, amitosis and mitosis, for the type of amitosis which I have described involves only the nucleus, and mitosis was the only process which resulted in the formation of two separate cells.

These observations upon nuclear amitosis do not point to its being an evidence of cell degeneration, for the cells in which it is found are not highly specialized and do not show any more tendency to degenerate than the other cells of the culture. It is generally assumed that mitosis takes place only in normal cells, so that the occurrence of mitosis in amitotically divided nuclei hardly allows them to be considered as degenerate. So, too, the occurrence of amitosis and mitosis in the same preparation (as in the culture from which figure 2 was drawn), where the conditions under which the cells are growing are apparently identical, militates against the view that the environment is not favorable, for the two processes are going on side by side, and mitosis demands suitable conditions. The statements of Wieman (1910, p. 174), "amitosis occurs usually under abnormal metabolic conditions which are unfavorable to normal metabolic processes" and "it can occur under circumstances that make mitosis impossible," are out of harmony with his finding of both direct and indirect division side by side in the same field, as shown in his figure 13. This coincident occurrence of mitosis and amitosis has been noted by other investigators.

The conception of amitosis which I have advanced thus differs radically from that of Flemming (1892 and 1893), vom Rath (1891 and 1895), Ziegler (1891), and Ziegler u. vom Rath (1891). They believed that amitosis occurred in cells which were of a transient character and in those which were very highly specialized or on the way to degeneration; and that in cells of amitotic origin the process of mitosis was not believed to take place. In their scheme the condition which I shall speak of as nuclear fragmentation seems to have a place.

According to this conception, then, amitosis constitutes simply a change in form of the nucleus without increase in its reproductive capacity, and not an actual cell division; and division of such an amitotic cell occurs only by karyokinesis in which there is a recombination of the nuclear material. If this view be correct,

and of universal application, it may be possible to reconcile amitosis with the chromosome hypothesis, for, since mitosis would be the only method of actual cell proliferation, an unequal distribution of chromatin material to the daughter cells would not be possible, according to our conception of the mitotic process.

NUCLEAR FRAGMENTATION.

A note may here be made regarding a curious form of nuclear division which bears some resemblance to the one just described, but which differs from it in many important particulars. It is known as nuclear fragmentation or unequal multiple nuclear fission, and was found to occur where the conditions for growth were not favorable—for instance, in old cultures, in which the food and oxygen supply had become depleted and katabolic products had accumulated (figs. 36 to 47) and in those to which a toxic constituent had been added (*e. g.*, ethyl alcohol, figs. 48 to 58). It thus seems to be a pathological condition and is characterized by marked malformation of the nucleus, manifesting itself in lobulation and by a breaking away of these lobules, so that what was formerly a single nucleus comes to consist of two, three, or as many as seven or eight apparently separate pieces.

The forms in which fragmentation presents itself are various, as may be seen by reference to figures 36 to 47, drawn from a 6-day growth from the stomach of a 5-day chick. The nucleus may be but moderately deformed, as in figure 49, where a small bud has become constricted off, or there may be two, three, or more lobes or appendages, as seen in figures 40 and 41. These small fragments are in all stages of constriction, ranging from a blunt, sessile protuberance to a small pedunculated mass, held sometimes by a mere thread, as in figure 46. Extremely irregular forms, as 37, are not infrequent, and completely separated portions, as in 36, 42, and 45, are quite often met with. Each fragment may or may not contain a nucleolus. In the smallest pieces it is absent. In some cases, as in figure 45, if the nucleolus happens to be caught in the constricting zone it may become separated, but this is a rare occurrence. Where the nucleus is lobulated the number of lobules usually exceeds the number of nucleolar portions. The culture shows other evidences of degeneration. The size of the nuclear portion seems here to bear no relationship to the size of the karyosome fragment, as it does in the multiple direct division of the nucleus described by Schultz (1915).

The extent to which this process of fragmentation may proceed is seen by reference to the fact that 66 per cent of the nuclei were malformed in some way, and 34 per cent were actually fragmented, in ten fields from the preparation from which figures 36 to 47 were drawn. There were no mitotic figures found in this preparation.

In no case was there found any evidence of division of the cell protoplasm following nuclear fragmentation; on the contrary, a sort of syncytium was formed, in which the cytoplasm was filled with nuclear fragments of varying size. The picture presented by such a nuclear complex is markedly different from that of the giant cell, among the points of differentiation being the widely varying size of the nuclei, their lobulation, and the presence of buds in process of separation from the main nuclear mass. Again, in fragmentation the cytoplasm does not increase, as in the case of the giant cell.

The entire absence of division of the cell protoplasm prevents this nuclear change from being regarded as a method of cell proliferation. Again, there is no evidence that such nuclear fragments ever reunite to form a spireme after the manner already described for the ordinary type of amitotic nucleus; indeed, mitotic figures are absent from such preparations—a fact which seems to indicate that the conditions which bring about fragmentation also prevent karyokinesis.

The differences which fragmentation presents as compared with the usual form of direct nuclear division may be briefly summarized as follows: The nucleus is of irregular contour, multilobulated, and breaks up into a number of small, unequal-sized parts, which frequently do not contain nucleoli; the nuclear parts remain small, indicating that they have little or no power of growth, for the total volume of the nuclear substance does not seem to be increased following division. There is no evidence of fusion of the fragments contained in a single mass of cytoplasm to form a single mitotic figure. Finally, the process is found in growths which are existing under abnormal conditions, such as the presence of toxins or a deficiency of oxygen, and such conditions act to prevent mitosis.

As contrasted with this we find, in the case of the ordinary binucleate or multinucleate cell, nuclear portions of regular contour, few in number (usually not more than two), of almost equal size, each containing as a rule one or more nucleoli. These parts apparently possess the power of growth, for in size they are comparable with the nuclei of the mononucleate cell. The fragments of the "double" nucleus are also able to combine and form a single mitotic figure. These cells are found in normal cultures, in which mitotic figures are frequently to be seen.

Fragmentation is similar to the division which produces the ordinary binucleate cell in that the position of the centrosphere and mitochondria with relation to the nucleus is the same. In figures 48 to 58 these structures will be seen occupying the cleft, as in 55 and 58, or situated between the fragments, as in 50 and 54.

Nuclear forms of this character are not infrequently found in the literature. Glaser (1907) describes an analogous form of nuclear fragmentation which occurs in the degenerating food ova of *Fasciolaria tulipa*. This he regards as "pathological amitosis" as distinguished from physiological amitosis. Child (1907*c*, p. 288) speaks of "degenerative amitosis" in starving planarians, stating that these forms "differ in appearance from the amitoses in regenerating tissues;" again (1907*c*, p. 173) he finds that "nuclear fragmentation is a frequent accompaniment of degeneration."

On the whole, therefore, judging from the prevailing views of authors, and from the conditions obtaining in the cultures in which it occurs, it seems reasonable to regard nuclear fragmentation as an evidence of degeneration. These final changes are, perhaps, to be looked upon as an active reaction of the nucleus to unfavorable conditions of its environment, as, for instance, the presence of toxins due to katabolism, or chemical change in the media, or injurious material added to the media, as alcohol, or to deficiency in food or oxygen.

In this connection it is interesting to note that Lewis (1911) and Miller and Reed (1912) demonstrated that the presence of toxins caused an increase in the number of lobes of the neutrophilic leucocyte in blood of the human subject and also in that of the guinea pig and rabbit. They looked upon this increase as a physiological reaction on the part of the leucocyte. Wherry (1913) found that amœbæ

grown in oxygen-poor media showed division of the nucleus without cleavage of the cell protoplasm, and Wieman (1910) expresses the view that lack of oxygen may be a cause of a similar nuclear fragmentation in the material which he examined. Again, Holmes (1914) noted such a fragmentation in tissue cultures kept a week or more without changing the medium; when, however, the medium was changed frequently there was no indication of such nuclear change. Fragmentation was accompanied by other evidences of degeneration. Here, too, lack of oxygen may be the underlying cause, and the increased nuclear surface due to the change in form and multiple division of the nucleus may represent the effort, on the part of the cell, to secure an increased respiratory area.

The mechanics of nuclear fragmentation is no less complicated than that of true nuclear amitosis; indeed, it is probable that new and obscure factors bring about a change in nuclear outline and division of its substance. The activity of the centrosphere and mitochondria may be regarded as similar to that found in the true form of nuclear amitosis, since their relation to the nucleus is the same.

Nuclear forms somewhat resembling those just described, but simpler in character, are occasionally seen in apparently normal tissue cultures; *e. g.*, those shown in figures 11, 12, and 13. Similar forms have been described in embryonic tissue developing normally, as figures 7*b*, 8 *a* and *c* of Child (1904) and some of the figures of Maximow (1908). They appear to be examples of sporadic and simple fragmentation. The fate of these buds is obscure, but is probably degeneration.

SUMMARY.

The following general conclusions, based upon the results of the foregoing investigations, have been reached:

BINUCLEATE CELL.

Incidence: In 20 preparations the binucleate cells made up 0.9 per cent of the total cells appearing in the new growth. They were more abundant in membranes growing from the heart than in growths from any other tissue, and in cultures of hearts of 5 days of age than in those from older cardiac tissue. They were also more abundant in new growths from cultures of the second day than in those of the first; this suggests that some, at least, of these cells have arisen in the new growth rather than in the original piece, with subsequent migration into the new growth.

The proportion of cells containing amitotic (constricted) nuclei to the total number of cells was 1 to 835; that of amitotic nuclei to bipartite nuclei was 1 to 7.5, and that of amitotic to mitotic nuclei was 1 to 3.4.

Origin: The paired nuclei of binucleate cells in tissue cultures arise by direct division of the nucleus, or nuclear amitosis, without division of the cytoplasm. This occurred in perfectly normal cells.

Constriction of the nuclear membrane, from one or both sides, which seems to be associated with a karyoplasmic streaming away from the nuclear equator, was the only mechanism observed in direct nuclear fission, and in this process an activity of the centrosphere and mitochondria, combined with elongation of the nucleus, appeared to be the principal factors. The centrosphere does not divide, nor do the centrosomes separate.

The process of nuclear amitosis is slow, excepting the final stage, which is rapid. There seems to be a critical point in nuclear constriction; before this point is reached the nucleus may return to its original form, but after it has been passed the cleavage of the nucleus proceeds rapidly, and results in two separate nuclear parts.

Division of the nucleolus is not an essential of amitotic nuclear division; it may, however, be concerned with the size of the nuclear fragments.

There is no evidence of a form of nuclear amitosis that depends upon the formation of an intranuclear membrane which subsequently splits. Such a structure is simulated by the apposed surfaces of the nuclear membranes of the parts of the nucleus of a binucleate cell, when they are in close contact. Sometimes, also, nucleoli, mitochondria, and inbending of the nuclear wall may resemble such a membrane.

Fate: There is no evidence that direct division of the cytoplasm follows direct division of the nucleus; thus amitosis is not a method of complete cell division, but is to be looked upon as a change in form of a healthy nucleus.

The regular process of mitosis may occur in binucleate cells. During this process the chromatin material from both nuclear portions is merged into one equatorial plate of chromosomes, the spiremes, which begin to arise separately in the two nuclear parts, joining together to form the chromosomes. Furthermore, this is the only kind of cell division which was found to occur in binucleate cells; they either divide by mitosis or remain as they are, without fission of the protoplasm.

The separate parts of the double nucleus have no reproductive independence (though they may have metabolic independence), and act as a unit in mitosis. Hence the reproductive capacity of the bipartite and monopartite nucleus is the same.

Mitosis occurred as frequently in the binucleate as in the mononucleate cells.

Nuclear fusion, without mitosis, has not been found to occur.

GENERAL.

Mitosis occurs in a nucleus irrespective of its shape; thus the spireme was found in nuclei of rounded form, in those presenting equatorial constriction, and in those divided into two portions.

Chromosome hypothesis: Nuclear amitosis is not incompatible with theories of inheritance which assume that the chromosome is the bearer of hereditary characters.

Giant cells: The binucleate cell seems to be the first stage in the formation of the giant cell, which probably arises by a repetition of nuclear amitosis. This conception does not include the formation of the foreign-body giant cell.

Nuclear fragmentation was found to occur where conditions for life were not favorable, and was thus a form of degenerative change. Fission of a healthy nucleus (amitosis) must thus be distinguished from fission of an unhealthy nucleus (fragmentation).

Karyosomes of the cells examined were irregular in shape, underwent continuous change in morphology, size, number, and position, and were made up of numerous closely packed masses of gel, each with a core of greater density.

The centrosphere in the cells examined was a slightly concentrated gel containing a centrosome (usually paired). Its border is irregular, and this undergoes continu-

ous change in outline and appears to be intimately associated with adjacent mitochondria.

Vital dyes: Gentian violet did not prove to be a true vital dye. While it stained the intranuclear bodies and nuclear membrane, its action was toxic and coagulative, and the cells speedily degenerated.

Janus green, in low dilutions, was found to stain mitochondria specifically, but its action was destructive, causing speedy death of the cell, with dissolution of the mitochondria.

Embryonic cells: Many, at least, of the facts obtained from observation of cells in tissue cultures may be applied to the interpretation of similar cells developing normally in the embryo.

In conclusion I wish to record my indebtedness to M. R. and W. H. Lewis for the loan of their splendid collection of fixed preparations, and for their valued guidance; also to Dr. F. R. Lillie for the courtesy of a room at the Marine Biological Laboratory at Woods Hole, where some of the work was carried on.

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EXPLANATION OF PLATES.

PLATE I.

FIG. 1. Area of new growth from No. 42, 8 : 12 : 14 (Lewis collection). In the field are six binucleate (*a*) and one quadrinucleate (*b*) cells. The material was heart from a chick which had been incubated for 6 days; growth was of 48 hours' duration, in Locke (0.5 per cent dextrose), fixation by osmic-acid vapor, and staining with iron hematoxylin. (*t*) shows two young daughter cells, the product of a recent mitosis. The guide-line from (*a*) terminates in the centrosphere of a binucleate cell. Retouched photograph. ×465.

FIG. 2. Elongated nucleus with bilateral constriction—the beginning of direct bilateral nuclear fission. The nucleolus is also apparently dividing. This figure, and also Nos. 3, 4, 5, 11, 12, 13, 14, 15, 16, 17, 18, and 22 are from No. 14, 9 : 1 : 15 (Lewis). Heart from 6-day chick; grown in Locke (0.5 per cent dextrose) with a little yolk; fixed on third day of growth in Zenker; stained with Mallory's connective tissue stain. On account of the technique the cytoplasmic details are not represented. This and following drawings, except figures 24 to 35, were outlined by camera lucida. ×1,012.

FIG. 3. Elongated nucleus almost completely divided; final stage of direct bilateral nuclear fission. ×1,012.

FIG. 4. Nuclear fission completed; nuclear parts divided and lying in contact. ×1,012.

FIG. 5. Direct unilateral nuclear fission; initial stage. ×1,012.

FIG. 6. Direct unilateral nuclear fission; final stage; cell of connective-tissue type; nuclear parts connected only by the merest filament; centrosphere between nuclear sacs; mitochondria streaming across the narrow connecting strand. Drawn from preparation No. 2 (Lewis); 7-day chick heart grown for 5 days in Locke (1 per cent dextrose); osmic-acid vapor and iron hematoxylin. ×1,032.

FIG. 7. Nuclear fission completed; growth from heart membrane; cell similar to that shown in figure 4, but prepared to show the centrosphere and mitochondria; the single centrosphere contains two centrosomes; its position, opposite the line of contact of the two nuclear portions (below and to the right), is characteristic. No. 42 (Lewis) (see fig. 1). ×1,032.

PLATE II.

FIG. 8. Final stage of direct bilateral nuclear fission in a cell of connective-tissue type; shows the somewhat unequal nuclear parts joined by a very slender thread, apparently the attenuated nuclear membrane; overlying this are several strands of mitochondria, a similar relationship to that of figure 6; the larger nuclear sac contains two nucleoli; the smaller but one. A large centrosphere, from which many mitochondria radiate, is conspicuous. The entire cell is very thin and shows mitochondria streaming out into the processes. The morphology and arrangement of the mitochondria is characteristic for the connective-tissue cell growing *in vitro*, at periods other than mitosis. No. 17, 21 : 11 : 14 (Lewis). 6-day chick stomach; Locke (1 per cent dextrose); 3-day growth; osmic-acid vapor and iron hematoxylin. ×1,012.

FIG. 9. A binucleate cell from heart membrane; the two parts are somewhat separated, and lying between them a single centrosphere and mitochondria are to be seen; the latter resemble cocci or short bacilli and show the characteristic radia arrangement about the centrosphere. This type of mitochondria is found in cells of membranes growing from chick hearts. No. 42 (Lewis) (see fig. 1). ×1,012.

FIG. 10. Nucleus of distorted form in cell of connective-tissue type found in a culture growing in a weak alcoholic medium. The nucleoli in this preparation show as aggregations of granules; this appearance of the nucleoli in connective-tissue cells stained in this way is found when differentiation with iron alum is carried too far. Mitochondria are apparently uninjured. No. 23, 21 : 11 : 14 (Lewis). 6-day chick stomach grown in Locke (1 per cent dextrose) to which ethyl alcohol had been added to make approximately 1 per cent; 3-day culture; osmic-acid vapor and iron hematoxylin. ×1,012.

FIGS. 11, 12, 13. These figures show a simple degree of nuclear fragmentation. They were found in a culture in which the cells were otherwise apparently normal. In figure 11 the nucleus is constricted at one end; the larger portion contains two nucleoli of unequal size and irregular shape. In figures 12 and 13 the constriction is farther advanced. No. 14, Lewis (see fig. 2). ×1,012.

FIG. 14. Prophase of mitosis. Nuclear membrane and nucleoli are disappearing and skein is forming; cell not yet rounded. No. 14, Lewis (see fig. 2). This, and the four figures which follow it, represent the process of mitosis in a mononucleate cell. All drawn from the same preparation. ×1,012..

FIG. 15. Metaphase. Cell rounded and compact; processes drawn in; cytoplasm granular and stains very densely with hematoxylin; definite spindle with equatorial plate of chromosomes. ×1,012.

FIG. 16. Anaphase. The chromosomes have separated and the remains of the spindle may be seen as faintly defined streaks connecting the two aggregations of chromosomes; cytoplasm still densely granular and darkly staining, the entire cell contracted; cell-processes small and thread-like. ×1,012.

FIG. 17. Early telophase. Chromosomes less clearly marked, the chromatin masses breaking up. No evidence of nuclear membranes is to be seen. The cytoplasm is dividing, as shown by constriction about the equator. Markedly granular and darkly staining protoplasm. ×1,012.

FIG. 18. Late telophase. The two daughter cells are seen, separated and spread out thinly; protoplasm stains much more lightly; nuclei well defined and contain coarsely granular chromatin. In each daughter nucleus the beginning of a nucleolus is to be seen; but is very small compared with the size of this body at maturity. ×1,012.

FIG. 19. Late prophase of mitosis, in cell probably of connective-tissue type; two centrospheres at opposite poles; nuclear membrane has disappeared; spireme well marked; mitochondria short and thick. No. 42, Lewis (see fig. 1). Figures 20 and 21 are from the same preparation. ×1,032.

FIG. 20. Early prophase in a nucleus showing beginning direct unilateral fission; skein forming, nucleoli disappearing; centrosphere still single, situated in the fissure; mitochondria becoming shorter and thicker, and are intermediate in these respects between those seen in figure 8 and those of figures 19 and 21; nuclear membrane has almost disappeared. Cell of connective-tissue type. ×1,032.

FIG. 21. Late prophase in a nucleus undergoing direct unilateral fission. Skein has formed and nuclear membrane has disappeared; one centrosphere is to be seen in the cleft, and there is some indication of a second on the opposite side of the nucleus, in the area devoid of mitochondria. ×1,032.

FIG. 22. Prophase in a binucleate cell. Early stage. Skein is forming; membrane and nucleoli are disappearing. Some chromatin has become segregated in the area of contact between the two parts. The method of fixation and staining does not permit of the centrosphere and mitochondria being seen. No. 14 Lewis (see fig. 2). ×1,012.

FIG. 23. Prophase in a binucleate cell of connective-tissue type; somewhat later stage than figure 22. In each nuclear portion there has been formed simultaneously a skein and the nuclear membrane has disappeared. The chromatin material of the combined double nucleus will form a single equatorial plate of chromosomes, as in figure 67. Only one centrosphere, containing two centrosomes, is seen in the preparation, situated at one extremity of the fusing nucleus, it having come from the interval between the nuclear parts. It is thus probable that the spindle will form parallel with the long axis of the fusing nucleus. Mitochondria are short and thick. No. 18, 21 : 2 : 14, Lewis. 7-day chick heart, grown for 2 days in Locke (0.25 per cent dextrose); osmic-acid vapor and iron hematoxylin. ×1,032.

PLATE III.

FIGS. 24–35. A series of drawings from a living connective-tissue cell made at 15-minute intervals for 2¾ hours. The nucleus at the start was elongated and notched at one side. It was seen to take various forms, and ended as two separate nuclear parts. The series thus shows direct nuclear fission. It will be seen that the centrosphere is contained within the unilateral cleft, and when the nucleus ultimately divides the centrosphere is situated between the parts of the nucleus; mitochondria stream across the interval separating these two parts. The nucleolar bodies undergo interesting changes. The nuclear outlines, position of nucleoli and centrosphere, the cell outlines, and principal features of the cytoplasm were sketched in freehand from direct observation of the living cell. The drawings were afterwards retouched by reference to fixed preparations. Small circles represent fat globules, and short threads mitochondria. *c*, figure 24, marks the centrosphere. 5-day chick heart; 57 hours' cultivation, from No. 7, 9 : 1 : 15, in Locke (0.5 per cent dextrose) with extract of chick embryo. ×about 900.

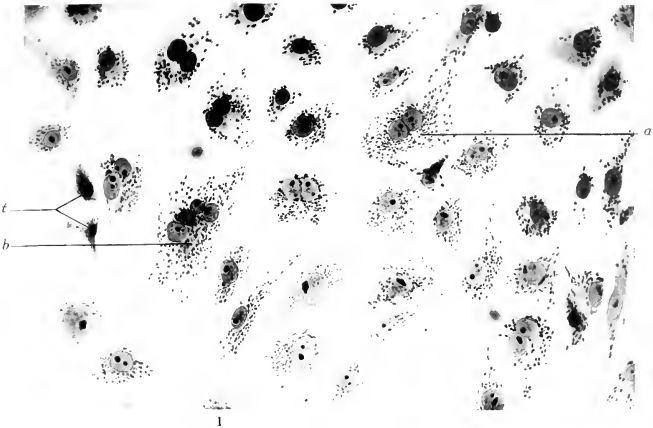
FIGS. 36–47. Fragmenting nuclei showing probable effect on form of nucleus of prolonged growth in unchanged media; outlines of nuclei very irregular, each has a number of lobes; in some cases separation of these lobes has taken place. Culture shows other evidences of degeneration. No division of the cytoplasm following division of the nucleus was observed. Drawn from various cells selected from No. 23, 12 : 1 : 15 (Lewis). 5-day chick stomach in Locke (0.5 per cent dextrose). Zenker; Mallory connective-tissue stain. Culture grown for 6 days in the same media. ×1,012.

FIGS. 48–58. A collection of nuclei of irregular form, grown in media containing alcohol; centrospheres are sketched in to show their characteristic relationship. Same preparation as figure 10. ×1,500.

FIG. 59. A regular paired nucleus from the same preparation as that from which the series 48–58 was drawn. Some of the nuclei have escaped distortion. ×1,500.

PLATE IV.

FIGS. 60–70. A series of camera-lucida drawings from a single living binucleate cell of the connective-tissue type, which was observed continuously for 8 hours. At the beginning there were two separate nuclear parts, with one centrosphere; the parts combined to form a single mitotic figure, and the successive stages of mitosis are seen in figures 66 to 70. The ultimate result is two separate mononuclear cells, each containing a single centrosphere. The series brings out the fact that the parts of the "double" nucleus are not independent so far as their reproductive capacity is concerned, but in cell division they combine and act as a single nucleus. *c*, figure 60, represents the centrosphere. 7-day chick heart, grown for 19 hours when the observation commenced. Locke (1 per cent dextrose) with extract of chick embryo. Culture of March 15, 1915. ×1,500.



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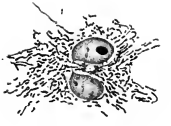

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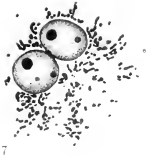

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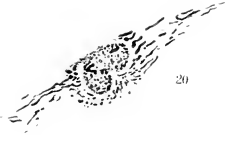
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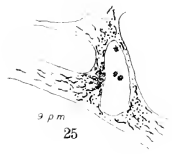

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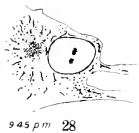

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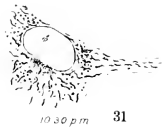

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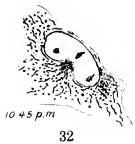

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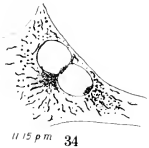

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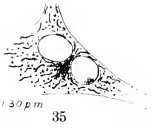

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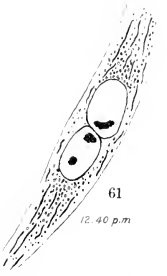
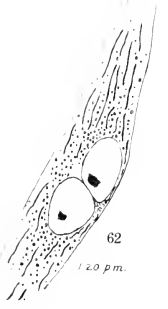


61 12.40 p.m



62 1.20 p.m.

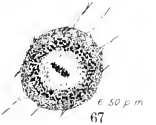
1.50 p.m. 63

64 3.05 p.m

65 5.05 p.m

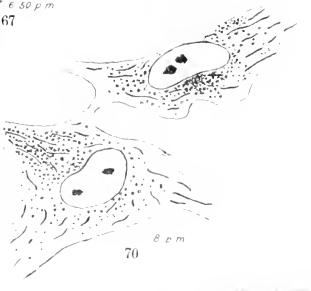
68 7.05 p.m

66 6 p.m.



6.50 p.m 67

69 7.25 p.m



8 p.m 70

M del